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EXPLORING NEW PEG-DENDRITIC BLOCK COPOLYMERS AS SIRNA DELIVERY VECTORS

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Resumo

Nas últimas décadas, a terapia genética emergiu como abordagem terapêutica pioneira para prevenir e tratar várias doenças. O conceito original de terapia genética baseia-se na introdução de fragmentos de ADN no interior de células com a finalidade de corrigir determinadas patologias. Mais tarde, este conceito tornou-se mais abrangente, passando mesmo a incluir outras estratégias como a regulação de genes. Dentro destas metodologias, a mais promissora é a regulação negativa da expressão de proteínas mediada por siARNs.

A terapia genética requer o desenvolvimento de veículos transportadores clinicamente adequados, seguros e eficientes, com a capacidade de compactarem e protegerem ácidos nucleicos, uma vez que estes, quando administrados sistemicamente, são altamente instáveis e vulneráveis à degradação por endonucleases presentes no soro. Até agora foram estabelecidas duas classes de veículos transportadores de ácidos nucleicos: virais e não-virais. Com o passar dos anos, os vectores não-virais têm vindo a ser preferencialmente escolhidos em detrimento dos virais, por aspectos relacionados com a segurança dos mesmos.

De entre os transportadores não-virais, os dendrímeros têm vindo a atrair um interesse particular devido às suas características estruturais únicas: estrutura globular bem definida, ramificações, a sua monodispersão, a presença de vários grupos funcionais terminais que podem ser funcionalizados de maneira específica e controlada, bem como a sua capacidade de complexar e encapsular ácidos nucleicos. Contudo, uma das maiores desvantagens dos dendrímeros mais usados é a sua incapacidade de serem degradados em condições fisiológicas, o que pode resultar em citotoxicidade induzida pela acumulação de materiais sintéticos não-degradáveis no interior das células e tecidos. Desta forma, estudos recentes têm-se focado no desenvolvimento de dendrímeros biodegradáveis.

Contudo, até agora, muito poucos estudos reportaram o uso de dendrímeros biodegradáveis como transportadores de ácidos nucleicos. Tanto quanto sabemos, não existem quaisquer estudos sobre a sua utilização na libertação de siARN. Recentemente, na nossa equipa foi sintetizada uma nova família de co-polímeros de bloco PEG-dendríticos, que demonstraram ser biocompatíveis e biodegradáveis. Este projeto foca-se na avaliação das suas performances como veículos de libertação de siARN.

Abstract

Over the last decades, gene therapy has emerged as a pioneering therapeutic approach to treat or prevent several diseases. The original concept of gene therapy was based on the simple idea that the transfer of exogenous DNA material into cells could serve as a therapeutic strategy to correct certain genetic disorders. Later, this definition became broader with the inclusion of other strategies such as gene regulation. Among them, one of the most promising methodologies is the down-regulation of protein expression mediated by small interfering RNAs (siRNAs).

Gene therapy requires the development of clinically suitable, safe and effective delivery vehicles with the ability to compact and protect nucleic acids since they are highly unstable and rapidly degraded by serum nucleases when systemically administered. So far, two classes of nucleic acid vehicles are clearly distinguishable: viral and non-viral. Lately, non-viral vectors are being chosen over their viral counterparts due to safety concerns.

Among the non-viral carriers, dendrimers have gained considerable attention due to their unique structural characteristics: well-defined globular and very branched structure, their monodispersion, presence of several terminal functional groups that can be functionalized in a specific and controllable manner, as well as their capacity to complex and encapsulate nucleic acids in compact structures. However, one major drawback of the most currently used dendrimers is their non-degradability under physiological conditions, which can result in cytotoxicity induced by the accumulation of non-degradable synthetic materials inside cells. Thus, recent research is being focused on the development of biodegradable dendrimers.

However, so far only a couple of studies have reported the use of biodegradable dendrimers as nucleic acid vectors. To the best of our knowledge, there are no reports on siRNA delivery. Therefore, this project studies the siRNA delivery performance of a new family of biocompatible and biodegradable PEG-dendritic block copolymers recently synthesized in our team.

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Index

List of Figures

List of Tables

List of Abbreviations

pDNA	Plasmid DNA
AONs	Antisense oligonucleotides
siRNA	Small interfering RNA
mRNA	Messenger RNA
RNAi	RNA interference
miRNA	Micro RNA
shRNA	Short RNA
Pri-miRNA	Primary micro RNA
RISC	RNA-induced silencing complex
PTGS	Post-translational gene silencing
NPs	Nanoparticles
PEG	Poly(ethylene glycol)
PAMAM	Poly(amido amine)
PEI	Poly(ethylene imine)
PLL	Poly(L-lysine)
G*n*	Dendrimer generation (*n* = 1, 2, 3 …)
Bis-HMPA	Poly(2,2-bis(hydroxymethyl)propionic acid
PPI	Poly(propylene imine)
GATG	Gallic acid triethylene glycol
bGATG	Biodegradable gallic acid triethylene glycol
DAPMA	N,N-di-(3-aminopropyl)-N-(methyl)amine
FBS	Fetal Bovine Serum
DMEM	Dulbecco's Modified Eagle's Medium
EDF	Extended Depth of Field

Chapter 1 - Introduction

1.1 - Gene therapy

The combination of the knowledge of the molecular mechanisms of disease and the dissection of the human genome holds a unique potential to revolutionize the way diseases are perceived and treated. Together with the development of new molecular and genetic tools, this knowledge can be used to develop new therapeutic strategies that target a broad spectrum of diseases, from cancer to immune and inherited disorders [1].

Over the last decades, gene therapy has emerged as a pioneering therapeutic approach to treat or prevent several conditions. Its underlying principles rely on the modulation of the host's gene expression, following by the introduction of exogenous genetic material into somatic cells [2, 3]. Gene therapy includes many different strategies depending on the type of genetic material being used. For instance, the insertion of new competent genes through plasmid DNA (pDNA) allows the compensation of the lack or non-functionality of a specific protein [3-6]. On the other hand, the insertion of antisense oligonucleotides (AONs) and small interfering RNA (siRNA) mediates the down-regulation of defective genes [3-6].

Despite all the research done so far, little advances have been made when it comes to approved clinical applications leaving much of its promises unfulfilled [7]. Different extra- and intracellular obstacles hamper the success of gene therapeutic approaches. Additionally, undesired side effects such as pathogenicity, immunogenicity and toxicity constitute potential risks [2, 3].

Nucleic acids are highly vulnerable to degradation by serum endonucleases when administered intravenously, which further hampers their already low cellular uptake [8-11]. After internalization, they are exposed again to degradation in the endosomal/lysosomal compartments and in the cytoplasmic environment [8-11]. For gene therapy strategies that require nuclear internalization, the nuclear membrane constitutes yet another barrier.

Accordingly, current research has been focusing on the design and development of a wide variety of carriers, able to protect nucleic acids and efficiently deliver them into cells with minimal toxicity [8-13]. So far, two classes of nucleic acid vehicles are clearly distinguishable: viral and non-viral [6]. Even though viral based vectors have been extensively studied, showing great gene transfection rates, the adverse effects such as mutagenesis and

immunogenicity are important obstacles still to overcome [14]. Furthermore, the low-scale production, storage difficulties, and nucleic acid limited packing capacity remain crucial limitations that raise concerns for their translation to the clinic and encourage the development of non-viral vehicles for nucleic acid delivery [8, 14].

1.1.1- Gene therapy strategies

Nucleic acid therapies are divided into three main strategies. The classic approach is based on the delivery of exogenous genes in the form of pDNA [1]. Once inside the nucleus, pDNA will use the cellular machinery to express the target gene. Additionally, pDNA sequence can be further modified to carry promoters, enhancers, splicing and polyadenylation sites that better modulate gene expression [15]. If effectively employed, this strategy could be used to treat diseases where there is lack of a specific protein or set of proteins. For instance, several works have already been done towards the delivery of Factor VIII, a key player in the blood clotting process [16, 17].

The second strategy involves the powerful down-regulation of specific deregulated genes actively producing excessive amounts of deleterious proteins. This can be achieved by employing two different agents: AONs and siRNAs [4, 5]. Even though both act by hybridizing to a complementary sequence of messenger RNA (mRNA) and therefore inhibiting translation, their downstream mechanisms display several differences need to be further elucidated [4, 5].

AONs are synthetic single-stranded DNA sequences composed of 13 - 25 nucleotides [4, 5, 18]. These small DNA fragments were shown to efficiently bind to mRNA sequences thus inhibiting translation and protein synthesis [19]. Furthermore, some AON were shown to effectively inhibit the ribosomal access and movement along de mRNA by steric hindrance (Figure 1.1a) [4]. An alternative mechanism for AONs is the RNAse-H mediated cleavage of the mRNA sequence in the RNA/DNA duplex (Figure 1.1b) [6, 20, 21]. While the mRNA sequence is degraded, the oligonucleotide fragment remains intact and becomes available to bind another complementary mRNA [18, 20]. Regardless of the mechanisms triggered by the AON, specific silencing efficiency is strictly dependent on the AON sequence and subsequently binding affinity to the mRNA. Therefore, a careful and well-thought design of the AON sequence together with a good knowledge of the target mRNA is essential to obtain maximum silencing activity [5, 6, 22].

Non-modified AON fragments present low stability and cellular internalization due to the phosphodiester linkage between the nucleosides [5, 20, 23]. To overcome these drawbacks, without neglecting the silencing efficiency, researchers have been aiming towards the chemical modification of these molecules. The first modification was described in the late 80's with the introduction of the phosphorothioate linkage between nucleosides and the 3'- and 5'-terminal modifications with 2′-*O*-methoxyethyl (2′-MOE) or 2′-*O*-methyl (2′-OMe), which enhanced bioavailability and stability by increasing the resistance to endonucleases [24]. However, more recent studies confirmed that phosphorothioate backbone induces severe immunostimulatory responses through interactions with Toll-like receptors [5, 25]. Additionally, phosphorothioate antisense oligonucleotides led to renal tubule changes and thrombocytopaenia [5].

More recently has emerged a new category of oligonucleotides called splice-switching oligonucleotides, which are able to interfere with pre-mRNA and redirect splicing pathways [5]. This mechanism can be employed to abrogate incorrect or undesirable splicing or to produce specific and therapeutic mRNA splice variants [5, 26].

For most AONs, the interaction with serum proteins, immune cell activation and complement activation constitute some of the most common sequence-independent adverse effects which are usually dose-dependent. Hence, cytotoxicity and pharmacokinetic studies are essential to define toxic doses and enhance therapeutic effects. Additionally, sequence-dependent toxicity is also associated with "off-target" hybridization, which can be further minimized by bioinformatics studies [4]-

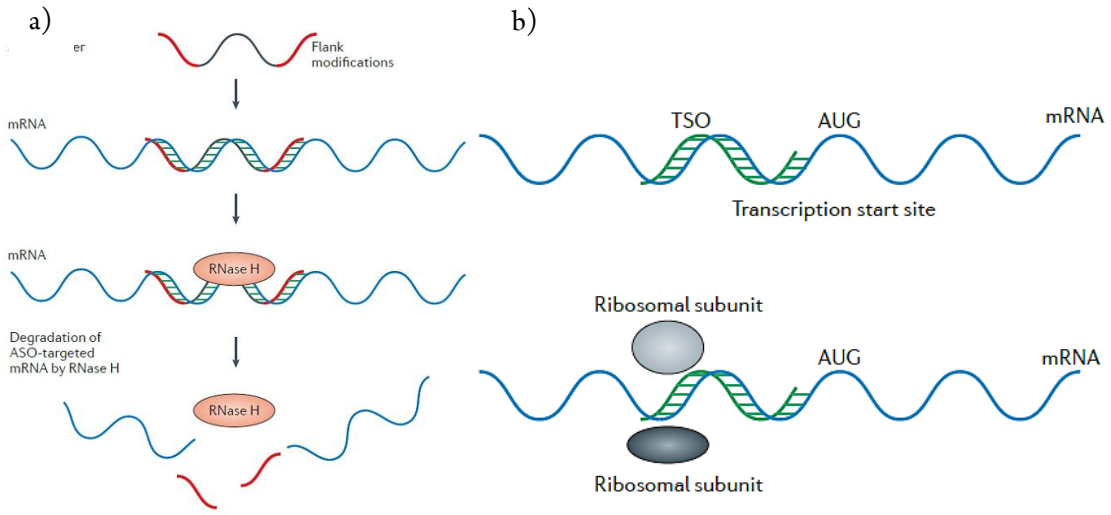


Figure 1.1 - Mechanisms of oligonucleotide-induced gene expression down-regulation. Gene expression down-regulation via: a) RNase-H mediated degradation of mRNA. b) Steric hindrance of ribosome access *to mRNA. (Adapted from Kole* et al., *2012) [5]*

RNA interference (RNAi) is a double-stranded RNA triggered process that was found to naturally occur in *Caenorhabditis elegans [27]* and later in mammalian cells [28]. Its mechanistic pathways were rapidly investigated, in an attempt to explore its potential for therapeutic purposes [10].

Briefly, RNAi is a post-transcriptional regulatory mechanism guided by double stranded RNA (dsRNA) derived molecules, namely micro RNA (miRNA) and siRNA. They are processed from endogenously expressed transcripts, and are able to trigger different downstream pathways that lead to translation arrest and/or degradation of homologous mRNA sequences (Figure 1.1) [10, 29, 30]. The first class of small RNAs, miRNA, is initially expressed as part of an imperfect RNA hairpin of ~80 nucleotides in length that forms part of a longer initial transcript termed a primary miRNA (pri-miRNA) [30]. Over 300 miRNA are found in the human genome and thought to have a major influence in both differentiation and development [31]. The second class, siRNAs, are either processed from longer dsRNA precursor molecules or exogenous small hairpin RNA (shRNA) [30, 32]. Despite the structural differences both long dsRNA and pre-miRNAs are processed by the RNase III enzyme Dicer in the cytoplasmic compartment [22, 33]. Briefly, after Dicer processing, the resulting small RNAs duplexes are both loaded into Argonaute protein and RNA-induced silencing complex (RISC) [10] or RNA-induced transcriptional silencing in the case of miRNA [30, 34]. Once within these complexes,

both siRNA and miRNA will recognize full or partial complementary mRNA sequences leading to post-translational gene silencing (PTGS), either by translation arrest or mRNA degradation [10, 22, 23]. Predominantly, miRNA initially leads to translational arrest and then non-sequence-dependent mRNA degradation in processing bodies [35], whereas siRNA essentially acts by triggering the sequence-specific cleavage of homologous mRNA [22, 30]. Interestingly, some studies have reported that miRNA can also induce RISC-mediated cleavage of mRNA transcripts, which generally occurs when there is complete sequence homology [10].

Several mechanistic aspects of this pathway have been extensively studied aiming at the discovery of new drug targets and tools for biological research. It is possible to exploit this endogenous mechanism in one of two ways: either by the introduction of exogenous vectors expressing small RNAs that mimic the endogenous pre-miRNA or by the introduction of siRNA fragments that directly proceed into Dicer-mediated processing, or direct RISC loading [36, 37]. Only recently, studies have stressed the efficacy of RNA-based strategies [22, 23]. By now, some proposed therapies have completed phase I clinical trials while others already proceeded into more advanced evaluation [38]. For instance, in 2009 a siRNA-based therapy targeting age-related macular degeneration completed phase I/II clinical studies [9, 38]. More recently, a siRNA-based therapy for the treatment of advanced tumors has finished phase I clinical trials showing promising anti-tumor activity [39].

In the present work, special attention will be given to the role of siRNA as a therapeutic agent, the mechanisms that it triggers, delivery barriers and recent findings.

1.1.2- siRNA-based therapeutics

The objective of RNAi-based therapeutics is to selectively induce gene silencing, either by translation arrest or mRNA cleavage. Gene silencing by mRNA cleavage is thought to be particularly powerful due to a hypothesized catalytic turnover [40]. The mRNA degradation process is believed to occur in a cyclic manner: as one mRNA fragment is degraded, the activated RISC targets intact mRNA strands and restarts the catalytic process [40].

Endogenous genes can be targeted by exogenous introduction of siRNAs, which then take advantage of the endogenous PTGS mechanism. Once in the cytoplasm the siRNA duplex is loaded into the multiproteic catalytic Argonaut/RISC complex. Here, the Argonaut protein detaches the so-called passenger (sense) strand from the guide (antisense) strand, and further cleaves the first. Afterwards, the intact guide strand activates the RISC that targets and cleaves complementary mRNA fragments between the 10^{th} and 11^{th} nucleotides (counting from the 5'-end) of the antisense strand (Figure 1.2). Subsequently, as mentioned above, the activated RISC can further search and degrade other complementary mRNA sequences, which adds an extra silencing efficiency, due to catalytic turnover [40]. It was shown that the large silencing capacity can last for 5-7 days in rapidly dividing cells and for several weeks in non-dividing cells [41].

RNAi approaches based on the introduction of exogenous shRNA, an analogue of the endogenous pre-miRNA, rely on the cellular machinery that processes miRNA to achieve gene silencing, which can be somewhat deleterious to the cell [36]. Studies have shown that the introduction of both vectors expressing shRNA led to tissue damage and death due to the saturation of the miRNA processing machinery, namely Exportin-5 [42]. Exportin-5 is a karyopherin that mediates the nuclear export of both pre-miRNA and shRNA [43, 44]. The

continued expression of high levels of shRNA in livers of adult mice resulted in the death of almost 50 % of the animals, which was attributed to the competition and subsequent saturation of Exportin-5 that ultimately led to liver-derived miRNA down-regulation [42]. Accordingly, overexpression of Exportin-5 was shown to both reverse the effects of miRNA down-regulation by shRNA and improve the efficacy of the RNAi-mediated gene silencing [44]. An additional advantage of siRNA-based therapies is that synthetic siRNA duplexes do not require nuclear export, as they are directly processed in the cytoplasm and therefore are less likely to interfere with endogenous RNAi pathways [30]. Moreover, siRNAs also bypass Dicer processing [9, 36]. Consequently, the majority of the RNAi-based therapeutic strategies developed so far propose the introduction of synthetic siRNA duplexes that target the final stages of the RNAi pathway.

Despite the great advances made in the last years on the understanding and harnessing of the RNAi-mediated gene silencing mechanisms, for the exploration of new drug targets and therapeutics, important obstacles are yet to be overcome in order to translate RNAi-based therapeutics into the clinics [36, 38]. On this subject, several studies highlighted non-specific and undesirable effects triggered by siRNA-based therapeutics, namely "off-target" silencing and activation of the interferon system [45-49]. Recent discoveries suggest that siRNA can function as miRNA and subsequently regulate untargeted genes [50]. By sequence analysis it was found that "off-target" silenced genes had partial complementarity with the guide strand of siRNA at the 5'-end [48]. Interestingly, later studies found that this fragment of the guide strand was equivalent to the 5'-end of an endogenous miRNA that mediates gene silencing by translation arrest and mRNA degradation [48]. Moreover, gene expression profiling studies using different siRNAs targeting the same gene showed that unique expression profiles were observed for each siRNA in concentration-independent manner [50].

"Off-target" effects are also visible through the induction of innate cellular defense that naturally evolved as an antiviral mechanism working through recognition of long exogenous dsRNAs [45, 49]. Initial studies attributed the activation of the interferon response only to longer dsRNAs and discarded that the same mechanism could be triggered by smaller dsRNAs (siRNA) [51, 52]. In contrast, Sledz *et al*. showed that siRNA transfection induced interferon-mediated activation of the JAK-STAT pathway and further up-regulation of interferon-inducible genes [49]. Moreover, siRNAs were also prone to induce IFN-independent genes [45, 49].

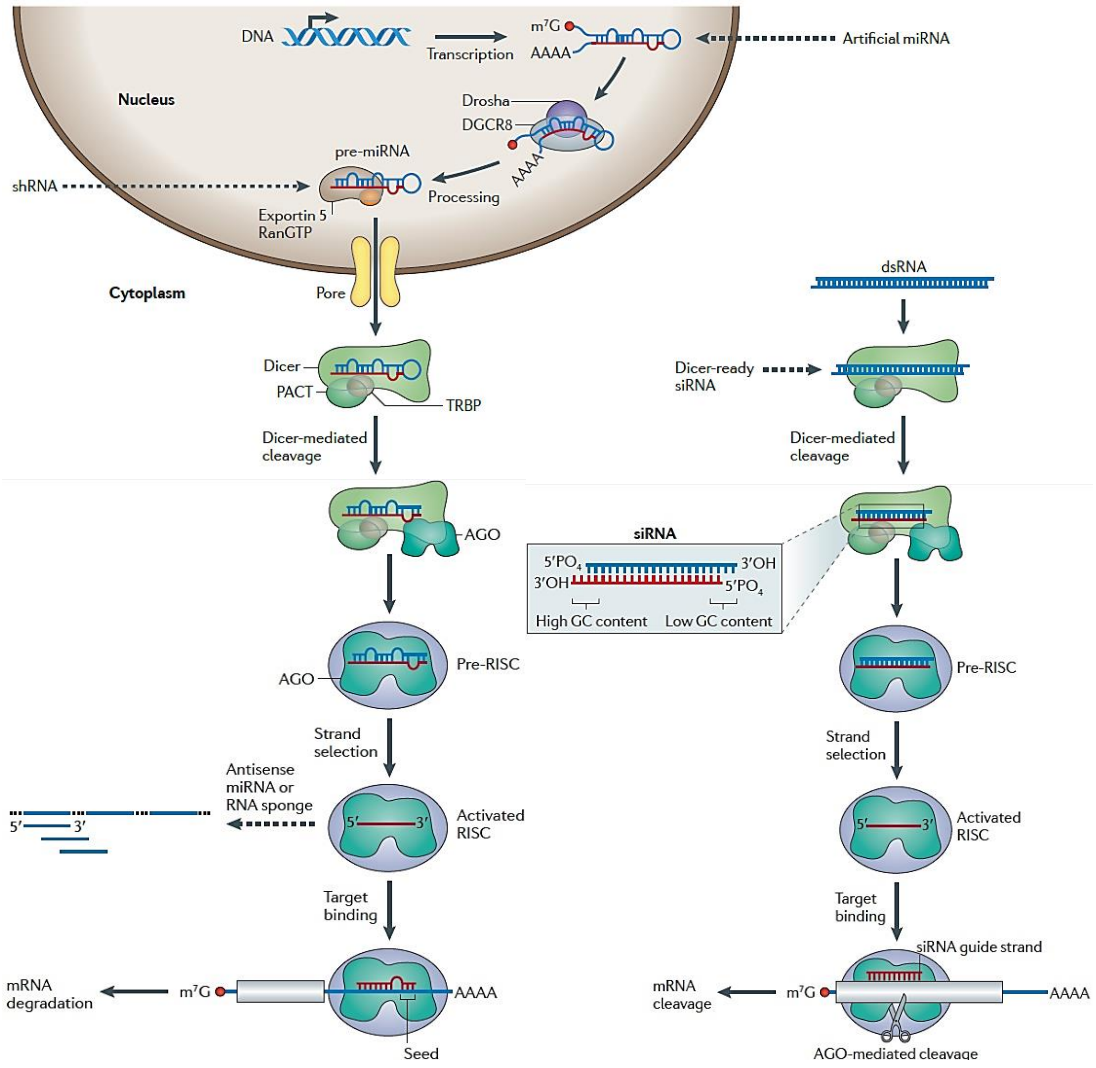


Figure 1.2 - The miRNA and siRNA pathways of RNAi in mammals. Primary microRNAs (pri-miRNAs) are transcribed by RNA polymerases and are cleaved by the protein Drosha into ~70 nucleotide precursors, called pre-miRNAs. Then, pre-miRNAs associate with the protein exportin 5 and are exported to the cytoplasm, where a complex that contains the protein Dicer processes the pre-miRNAs into miRNA duplexes. The duplex associates with an Argonaute (AGO) protein within the precursor RNAi-induced silencing complex (pre-RISC). One strand of the duplex (the passenger strand) is removed. The mature RISC contains the guide strand, which directs the complex to the target mRNA for post-transcriptional gene silencing. Long dsRNAs are processed by Dicer into siRNAs. Within the pre-RISC complex, an AGO protein cleaves the passenger siRNA strand. Then, the mature RISC, containing an AGO protein and the guide strand, associates with the target mRNA for cleavage. (Adapted from Davidson and McCray, 2011) [22]

Although this is not globally observable for siRNAs, the induction of immune response both *in vitro* and *in vivo* was found to be sequence dependent and attributed to specific immunostimulatory motifs within the siRNA fragments [46, 47]. Moreover, the induced immune response was further attributed to the type of delivery agent [46, 47, 49].

Until 2012, only 0.6 % of the clinical trials worldwide involved the use RNAi-based therapies [53]. Even though this therapeutic strategy is still in an embryonic stage, it is clear that the abovementioned issues are hampering translation to a clinical setting. Thus, it is extremely important to address these obstacles and develop more efficient and safer therapies. Chemical modification of the siRNA sequences is thought to reduce or even abrogate the "off-target" effects [38, 54]. In 2006, Jackson *et al.* reported position-specific and sequence-independent modifications able to reduce "off-target" silencing without

significantly compromising silencing efficiency of complete complementary mRNA [54]. Chemical modifications were also shown to reduce immunostimulatory effects [55]. Moreover, bioinformatics studies would help to rationally design and build more specific sequences, avoiding "off-target" silencing and activation of immune response [4, 36]. Additionally, further research into the mechanism of RNAi could give rise to new insights on gene regulation, allowing the development of better therapeutic applications and the demonstration of the efficacy and safety of RNAi-based approaches in a clinical setting.

Even though "off-target" effects and induction of innate immune responses constitute major concerns on the development of RNAi technologies, the aspect that has gathered more attention is the successful delivery of siRNAs [9, 10, 38]. To achieve an efficient gene silencing, therapeutic siRNAs have to overcome different extra- and intracellular barriers that vary with the administration mode [9]. Briefly, siRNAs can be directly administered into the target tissue or injected into the systemic circulation [9]. Local administration is relatively easy to perform in certain tissues like skin, mucus surfaces such as the lung and tumours [9]. Several studies have already reported the successful siRNA delivery to lung epithelial cells via intranasal route to treat respiratory syncytial virus and other diseases [56]. In 2007, Inoue *et al*. reported an electroporation method to deliver siRNA to the skin in an atopic dermatitis mouse model [57]. Interestingly, local administration at the hippocampus [58], and heart [59] was also performed to down-regulate alpha-synuclein and a sodium/hydrogen exchanger, respectively.

1.2 - Vectors for NA delivery

As already mentioned, gene therapy requires the development of clinically suitable, safe and effective delivery vehicles with the ability to compact and protect naked nucleic acids.

While some tissues are easily accessible allowing NA delivery by minimally invasive or non-invasive methods, other tissues are only safely and efficiently accessible through systemic administration.

Previous studies reported that, when intravenously administered, nucleic acids face different barriers before they reach the cells. Naked nucleic acids are highly susceptible to degradation by endonucleases and can further aggregate with serum proteins such as albumin, thus reducing an already low cellular internalization due to their inherent negative charge [8-11]. Moreover, following cellular uptake, unprotected nucleic acids are prone to additional degradation in the endosomal/lysosomal compartments and in the cytoplasm if the unlikely endosomal escape occurs [8]. Together, these obstacles decrease the genetic payload available, which then fails to trigger the desired response [8]. Consequently, over the last decades many studies have reported the development of nucleic acid delivery vehicles that, in theory, should help to overcome the different extra and intracellular barriers, in order to efficiently deliver them into cells with minimal toxicity [8-13].

So far two main categories of NA have emerged: viral and non-viral vectors [3, 8]. Viruses have naturally evolved to be highly specialized biological machines in cellular internalization and gene delivery [14, 60]. Within the cell, viruses work by using the cellular machinery to replicate themselves and express pathogenic genes, which in several cases causes deleterious

effects and ultimately cell death [3]. Thus, the rationale behind viral vector design for NA delivery is based on the removal of viral components responsible for pathogenicity while leaving intact the necessary components for gene assembly into the virus capsid or the integration into the host genome [3, 14, 60]. Thus, it exploits the viral infection pathway to deliver the target genes while avoiding toxicity [14].

Viral vectors, which were used in ~64 % of the clinical trials until 2012 [53], can be further subdivided into oncoretroviruses, lentiviruses, adenoviruses and adeno-associated viruses, depending on the morphology and delivery mechanism [7, 14]. Despite the high transfection rates, several hurdles concerning the production and viability of these vectors are slowing clinical translation. One major technical challenge starts at the production and purification of viral vectors [14, 61]. Meticulous procedures have to be employed in order to completely remove pathogenic components, and further purification is needed to obtain "safe" vectors, which implies high costs and hampers the process scale up [14, 61]. Another technical issue encountered in viral vector design is the limited amount of genetic payload that viruses can carry and delivery into cells [7, 14]. Last but not least, a large number of severe adverse effects such as immunogenicity and carcinogenesis have been observed for viral-based therapies [7, 14]. For instance, in 1999 the death of a 18-year-old man was attributed to an inflammatory reaction to an adenovirus-based treatment against ornithine transcarbamylase deficiency [62]. In 2000 Cavazzana-Calvo *et al*. reported the first definitive cure by gene therapy. Initially, 4 out of 5 children with human severe combined immunodeficiency were treated with a retroviral-based therapy, in which the successful insertion of the gene encoding γc chain receptor into CD34+ cells was achieved [63]. The γc chain receptor is a key player in the response to cytokine signals and also influences T and NK lymphocytes differentiation [63]. However, 3 years after treatment, 2 children developed T cell leukemia, which was later proven to be related with insertional mutagenesis of the gene near an oncogene [64, 65]. Later, new cases of T cell leukemia in patients that received the same treatment appeared and the trials were put on hold for safety and efficiency improvements [53]. Until now, new reports on successful treatments using viral vectors have been made for chronic granulomatous disease [66], metatastic melanoma [67], and Parkinson [68].

While some of the present research is focused on the optimization of viral vectors, the major safety drawbacks encountered by these have prompted the development of non-viral vectors, such as liposomes, linear polymers and, more recently, dendritic structures (dendrimers and dendrons) [6-8, 11, 69]. Non-viral vectors have already proven to overcome some of the obstacles encountered by viral vectors: they are usually easier to fabricate through innovative synthesis schemes, able to carry higher payloads of genetic material and also display lower immunogenicity [8, 11, 69, 70]. Following great advances in material sciences, a broad spectrum of non-viral agents for the delivery of nucleic acids have been reported [7]. However, the majority of them lack the transfection efficiency reported by the viral counterpart, which is slowing down their way to the clinic [7, 70]. Even though the delivery of naked nucleic acids by physical methods is widely considered a non-viral delivery strategy [13], here only vectors that result from the complexation of nucleic acids with other molecules are considered non-viral vectors.

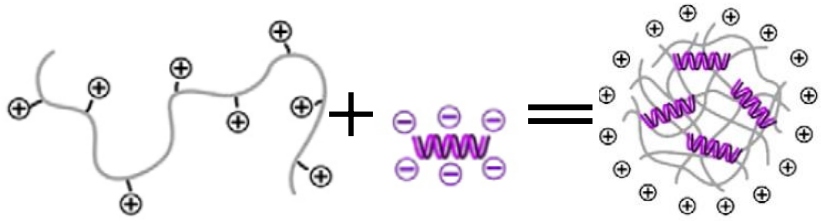


Figure 1.3 - Preparation of chitosan/Nucleic acid NPs. Positively charged chitosan binds to negatively charged nucleic acids via electrostatic interactions. (Adapted from Mao et al., 2010) [71]

Most of non-viral vectors are cationic, favoring complexation with the anionic nucleic acids through electrostatic interactions (Figure 1.3) [8, 11]. The nanoparticles (NPs) formed between nucleic acids and cationic lipids, polymers and dendrimers are termed lipoplexes, polyplexes and dendriplexes, respectively [11].

Unlike viruses, non-viral vectors are not naturally tailored to enter cells and deliver nucleic acids. Following intravenous administration they face a number of hurdles that can be overcome by careful design [7, 11]. Thus, recent progresses in materials science and nanotechnology have allowed the rational design of non-viral vectors towards a successful gene delivery [7]. The next section explains the different barriers to systemic delivery of nucleic acids and which strategies can be employed to avoid them.

1.2.1- Non-viral vectors and NA delivery barriers

The main hurdles towards a successful gene therapeutic intervention include: a) NA degradation by endonucleases present in the extracellular milieu; b) cellular internalization, c) endosomal escape; d) NA release from the vector and access to the cytoplasmic or nuclear target; and e) vector intra- and extracellular accumulation (Figure 1.4). Additionally, the delivery vector must avoid unspecific binding to serum proteins, preventing aggregation.

The first barrier is mainly avoided by the complexation of the nucleic acid with cationic materials that condense and protect nucleic acids into compact structures, obstructing the access of endonucleases to them [8, 70]. Condensation efficiency is strictly dependent on the material structure and its chemical properties. Another barrier encountered within the blood vessels is the interaction with other blood components such as salts and proteins. At high salt concentrations, the electrostatic repulsions between the cationic NPs will decrease, promoting destabilization and/or aggregation [7]. Additionally, the interaction with negatively charged serum molecules such as albumin promotes further aggregation and rapid clearance by macrophages [70, 72-75]. The most common strategy to improve serum stability is to combine the non-viral vector with “stealth molecules” such as poly(ethylene glycol) (PEG) [7, 8]. PEG is known to increase solubility and mask the positive charge of non-viral vectors, avoiding protein interaction and subsequent aggregation, hence improving blood circulation time [76-79]. Additionally, the length of the PEG chain has been shown to have major influences on the physicochemical properties of the entire complex [80, 81]. Other polymers such as poly(2-methyl-2-oxazoline) [82, 83], poly(2-ethyl-2-oxazoline) [82, 84], and poly(N-vinyl pyrrolidone) [85] have been reported as alternatives to limit protein fouling.

Cellular internalization is highly dependent on three main properties of the NPs: surface charge, size and targeting ligands [8, 11, 70]. Several studies have reported that non-specific cellular uptake is mainly driven by electrostatic interactions between negatively charged cell

membrane molecules, namely proteoglycans, and the NP surface [8, 11, 70, 86]. Baldeschwieler et al. showed that the removal of membrane proteoglycans severely hindered cellular internalization of cationic NPs [87]. Additionally, it was reported that cationic NPs present higher uptake rates than the anionic counterparts [86]. Conversely, particles with higher positive densities are known to induce more cytotoxicity [8]. Upon the interaction with the cell membrane, the NPs are likely to undergo different uptake mechanisms: clathrin- and caveolae-mediated endocytosis, phagocytosis, macropinocytosis and both clathrin- and caveolae-independent mechanisms [11, 86]. Once NPs are spread along the membrane, their size has a major influence on the uptake pathway [11, 86]. For instance, clathrin-mediated endocytosis is able to internalize particles from 10 nm to 300 nm,[88] while caveolae-dependent mechanism only supports particles up to 100 nm [89]. Even though macropinocytosis and phagocytosis allow the internalization of particles up to and bigger than 1 μm, respectively, these pathways are rare in cells other than phagocytic cells [70, 86]. Unfortunately, these mechanisms are thought to occur simultaneously, being hard to study and identify which one is actively mediating NP internalization[90].

In the last years, a lot of research focused on the development of non-viral vectors with target ligands that take advantage of strong ligand-receptor interactions, promoting specific delivery by receptor-mediated endocytosis, thereby enhancing efficacy and preventing off-target effects [11, 12, 86]. Ligands can include antibodies, antibody fragments, proteins, peptides and other molecules [75]. Even though several molecules such as transferrin [91], folate [92], and asialoglycoprotein [93] have already been employed in receptor-mediated endocytosis, an efficient targeted delivery is not always straightforward and easy to achieve [86]. Once several specific and nonspecific uptake mechanisms are operating simultaneously *in vivo,* the success of each strategy relies on the adequate choice of the ligand-receptor pair, tissue specificity, and uptake capacity [86].

After internalization, NPs travel along the cytoplasm inside endosomes [8, 70]. The endosomal pathway is known to evolve through pH acidification, where the transition from early endosome to late endosome is marked by a rapid acidification due to the activation of the ATPase proton pump [8, 70]. Later, in the lysosomal stage, the pH drops to 4.5 together with the recruitment of degrading enzymes, creating a rather hostile environment for both the carrier and the nucleic acid [8, 70]. Additionally, new insights on the early stages of the endosomal pathway have described two types of early endosomes: the sorting and the recycling endosome [94]. While sorting endosomes evolve to late endosomes and ultimately lysosomes, recycling endosomes transport their cargo back to the cell membrane where exocytosis occurs [94]. Together with endosomal/lysosomal degradation, the recycling pathway has been shown as an additional barrier to efficient nucleic acid delivery [95, 96]. Thus, endosomal escape has been a subject of debate and study over the last years.

Cationic lipids are thought to mediate endosomal escape through fusion and lipid exchange with the endosomal membrane, which promotes membrane destabilization and, eventually, nucleic acid release [8, 70]. On the other hand, for cationic polymers and dendrimers two different mechanisms have been proposed [8]. The so-called “proton-sponge effect” describes the endosomal escape for polyplexes/dendriplexes with low pKa amine groups (about 5.5-6) [8, 70]. Upon influx of protons, these groups will become protonated, which causes an extra and extensive influx of protons, ions and water into the endosomal compartment, delaying acidification and further promoting swelling and membrane disruption by osmolysis [8, 97].

An alternative hypothesis describes the physical interaction between the endosomal inner membrane and the polyplex/dendriplex, which is thought to induce membrane permeabilization, and hence nucleic acid release [8]. Additionally, this theory states that there is no complete disruption of the endosome nor release of intact polyplexes/dendriplexes [98]. Instead, it describes a complete discharge of the encapsulated nucleic acid into the cytoplasm [98].

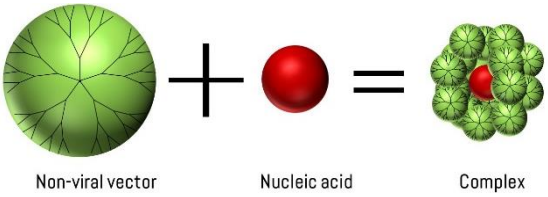
Consequently, different strategies have been proposed to overcome and enhance endosomal escape. For instance, Park *et al*. reported that the conjugation of histidines to a poly(amido amine) (PAMAM) dendrimer increased its buffering capacity, and hence endosomal escape [99]. Likewise, an enhanced buffering capacity was also observed through the conjugation of imidazole and hydrophobic moieties to chitosan [100].

Moreover, fusogenic viral or endosomal lytic peptides were also attached to NPs to enhance endosomal escape by membrane disassembly [8, 101]. Fusogenic peptides mimic the fusion of viral envelopes with host cell endosomal membranes [101].

Considering that the non-viral vector, either lipid, polymer or dendrimer, is released to the cytoplasm, the entrapment of the nucleic acid within the NP constitutes an additional barrier [8]. Therefore, efforts are being put into the development of vectors with cleavable linkers prone to stimuli-triggered degradation such as photo- and pH-sensitive, redox reactive and enzymatically degradable groups. While photo stimulation requires the use of exterior yet non-harmful stimulation, other linkers are susceptible to degradation in physiological environments [11]. For instance, the introduction of disulfide bonds between chitosan chains resulted in both enhanced pDNA displacement from the polymer and transgene expression [102]. Disulfide linkers are widely used for the design of non-viral gene delivery vectors due to their enhanced degradability in the reductive environment of the cytoplasm [103, 104]. Similarly, a few studies reported that the introduction of degradable bonds into the dendrimer shell was shown to enhance pDNA release profiles and/or transfection efficiency [105-109].

After endosomal escape and cytoplasmic release, nucleic acids must reach their target compartment. For siRNAs and AONs no further barriers are encountered as their biological targets are located in the cytoplasm [8]. In contrast, other nucleic acids, such as pDNA, must travel across the cytoplasm and through the nuclear membrane [8, 70]. This mechanism is highly dependent on the pDNA size and shape. Circular pDNA has been reported to move faster than linear pDNA chains [8].

Last but not least, cytotoxic bioaccumulation has been one of the main drawbacks in non-viral gene delivery [110]. Non-biodegradable vectors such as PAMAM, poly(ethylene imine) (PEI) and poly(L-lysine) (PLL) are prone to toxic accumulation, particularly after repeated administration [110]. These macromolecules are thought to interact with the cell membrane by destabilization of the lipid bilayer promoting permeabilization, loss of cellular content and, ultimately, cell lysis [111]. Cytotoxicity was further demonstrated to be highly dependent on macromolecule chemistry, size and surface charge, and morphology [112-114]. Consequently, there is a necessity for the development of biodegradable vectors able to efficiently deliver nucleic acids without any toxic accumulation. Ideally, after nucleic acid release, biodegradable vectors should break into smaller fragments to facilitate cell removal by exocytosis and further proceed into body excretion. Moreover, biodegradability mechanisms can be exploited for controlled release of nucleic acids in the cytosol [110].



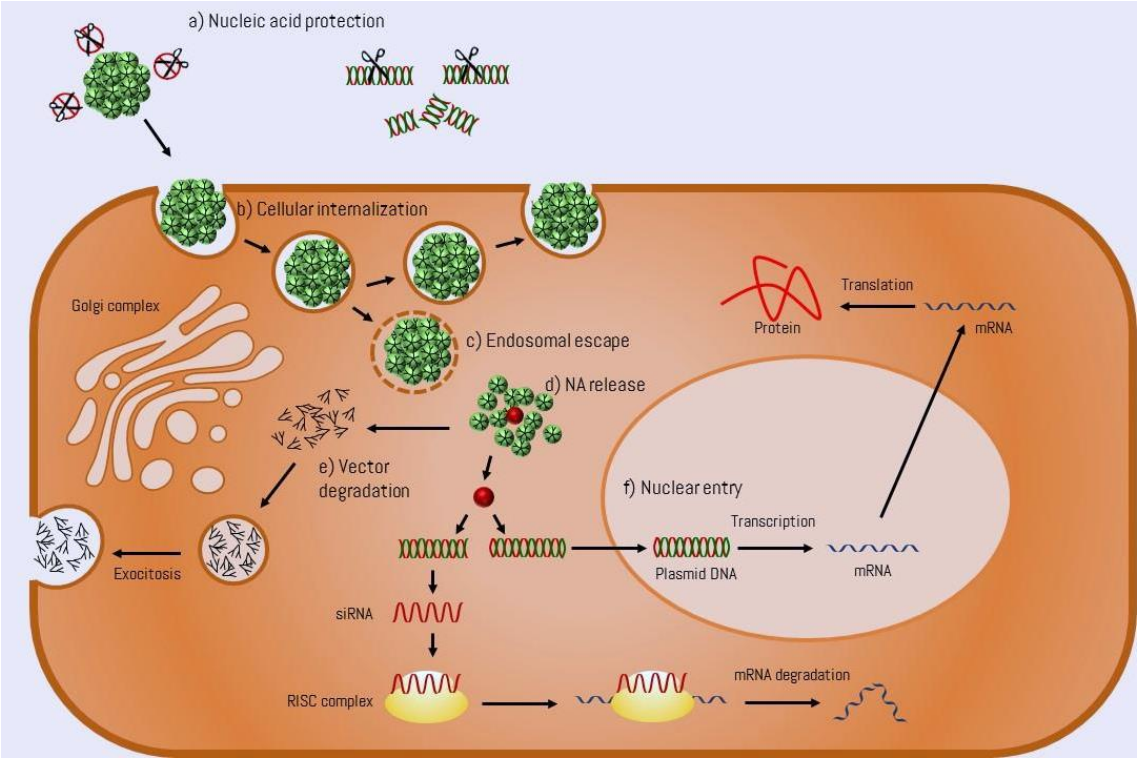


Figure 1.4 - Barriers for non-viral gene delivery. a) Protection against NA degradation by endonucleases, b) cellular internalization, c) endosomal escape, d) NA release from the vector and access to the cytoplasmic or nuclear target and e) vector degradation.

1.3 – Dendrimers

In the last 50 years, significant advances in materials science and polymer chemistry have contributed to the development of macromolecules with different architectures and compositions with great interest for biotechnological and biomedical fields [115-117]. One of the most recent development are globular and highly branched macromolecules called dendrimers [116]. Dendrimer-like structures were firstly synthesized by Voegtle in 1978[118] and further boosted by the groups of Denkewalter [119], Tomalia [120], Newkome [121], and Fréchet [122]. Since then, several reports have enriched this field with new dendritic structures, innovative synthesis schemes and different applications [116, 117].

Dendrimers are composed by three main components: the core, monomers covalently attached to the central core and organized in layers called “generations” and the surface (or peripheral groups) (Figure 1.5) [116, 117]. The core can assume different shapes (i.e. spherical or ellipsoidic) and its multivalency defines the number of branched monomers that can be attached to it [116, 117, 123]. Additionally, the core and number/type of branched

monomers are responsible for the tridimensional structure and the overall morphology and rigidity of the dendrimer [116]. Here, the dendrimer generation is defined as the number of focal points when going from the core to the surface [124]. When the core of the dendrimer is removed, a number of identical fragments called dendrons remain intact (Figure 1.5) [117]. Furthermore, the number of peripheral groups is directly proportional to the generation number.

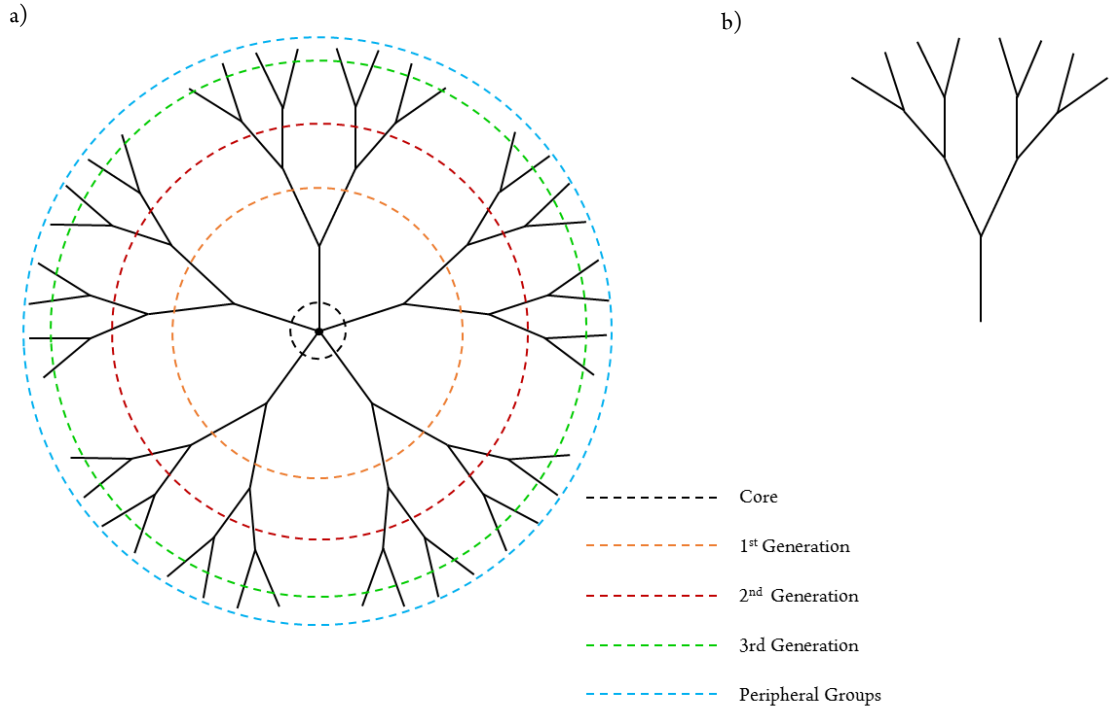


Figure 1.5 - **Dendrimer schematic representation. a)** Dendrimer; **b)** Dendron; Dendrimer's parts are represented by the dotted collored lines; Black: core; Orange: 1st Generation; Red: 2nd Generation; Green: Peripheral groups.

Some of the most known and widely used dendrimers are represented in Figure 1.6, including Tomalia's PAMAM [120], Denkewalter's PLL [119], Newkome's polyamide [121], Grinstaff's polyester (PGLSA-OH) [125], Vogtle's poly(propylene imine) (PPI) [118], and Hult's poly(2,2-bis(hydroxymethyl)propionic acid (bis-HMPA) structures [126]. Many of them are, in fact, commercially available such as the case of PAMAM, PLL and PPI, and have already been tested for different biomedical applications including gene delivery [92, 127], drug delivery [128] and magnetic resonance imaging [129, 130].

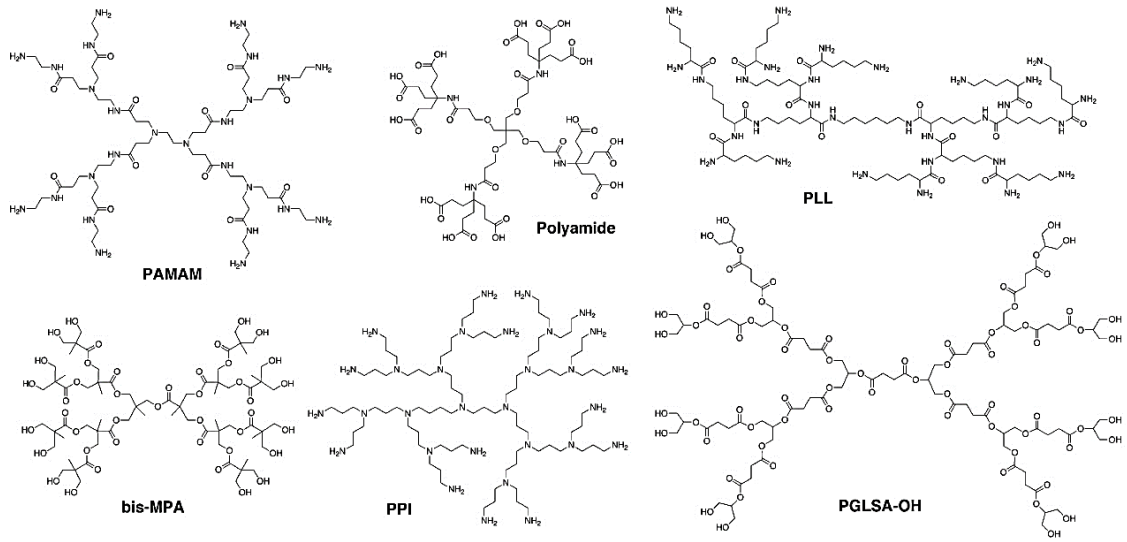


Figure 1.6 – Chemical structures of several commonly used and commercially available dendrimers. (Mintzer et al., 2010) [131]

1.3.1- Dendrimers: from synthesis to application

Much of the dendrimers' unique characteristics such as well-defined, globular and highly branched architecture, monodispersion and high density of peripheral groups arise from the stepwise synthesis strategies. Dendrimers can be synthesized using two conceptually different methodologies: convergent and divergent (Figure 1.7) [117, 123]. In the divergent approach, firstly described by Tomalia *et al.* [120], the multivalent core works as the starting point where monomers are assembled layer by layer. The dendrimer grows outwards, from the core to the surface [123, 131]. This stepwise process proceeds until steric hindrances between the peripheral groups prevent assembly of new monomers [123]. On the other hand, the convergent approach, established by Fréchet *et al.* [122], describes an inward growth process. Similarly to the divergent synthesis, the convergent method builds the dendrimer layer by layer, but starting from the terminal groups towards focal points, yielding individual dendrons [123]. Finely, to obtain dendritic structures, several dendrons are attached with a multifunctional core [116].

The divergent system is susceptible to defective monomer assembly due to the higher number of reactions performed at the same time, and thus, usually requires purification after each step [131]. The probability of byproduct formation increases with generation, which further results in lower purification rates [131]. To overcome these drawbacks, new user- and environmentally-friendly synthetic approaches have been reported, including cycloaddition [132, 133], thiol–ene chemistry [134, 135], and accelerated synthesis strategies [136], which resulted in higher reaction efficiency and step reduction [131]. On the contrary, in convergent synthesis only a limited number of groups is active per reaction, reducing the probability of structural defects [131]. The main drawback is its low ability to produce dendrimers of higher generations due to the steric hindrance between dendrons in the last step. An adequate choice of core size and multivalency can reduce steric hindrances [131].

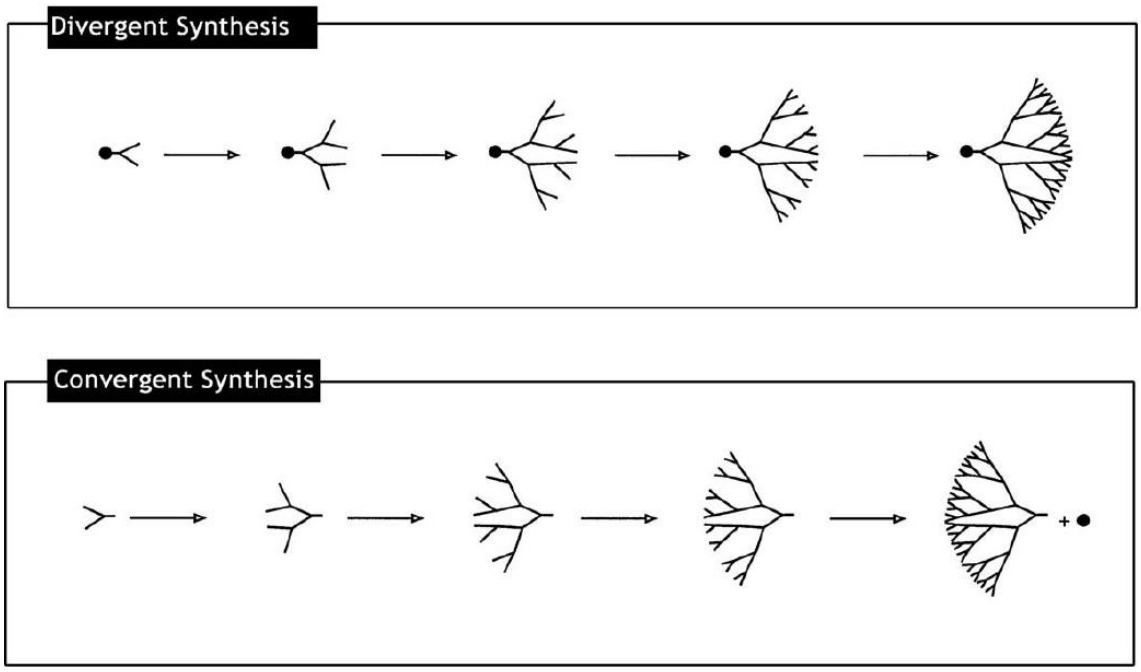


Figure 1.7 – Strategies for dendrimer synthesis. Divergent (top): dendrons are grown outwards starting from the dendrimer core; Convergent (bottom): dendrons are grown separately and attached to the dendrimer core in the final steps. (Adapted from Dufés *et al.*, 2005)[123]

In general, both strategies yield products with well-defined structures and monodisperse sizes, which is quite attractive not only for synthesis reproducibility but also for reducing experimental variability [117, 131]. Moreover, their adaptable chemistry allows to precisely design architectures with the desired physicochemical properties for different applications [117, 123].

The most attractive feature in dendrimers is their multivalency due to the high density of peripheral groups that allow multifunctionalization with different bioactive molecules/drugs. Multivalency has a great impact when it comes to biological or biomedical applications as the multiple simultaneous interactions with surfaces/molecules increase specificity and affinity [123, 131]. Moreover, dendrimers can be functionalized with several groups that can improve certain aspects of the dendrimer efficiency. For instance, the attachment of target molecules improves the dendrimer cell-specificity [123, 131], and the functionalization with PEG decreases toxicity, while increasing solubility and blood circulation times (Figure 1.8) [117, 123].

Additionally, both the core and branches represent well-defined nano-environments and have a great potential to act as nano-containers of a broad range of molecules, from drugs to biosensors [117, 123, 131].

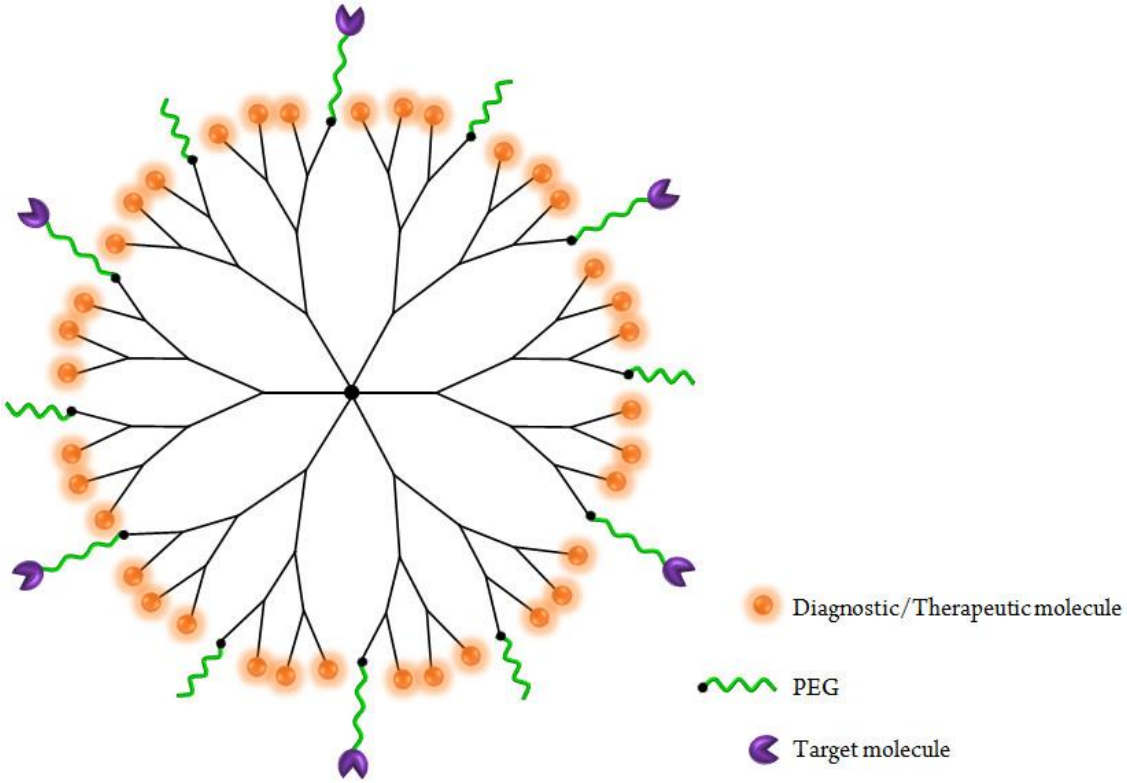


Figure 1.8 - Schematic representation of a targeted and PEGylated dendritic agent.

Besides the inherent characteristics of dendrimers, their *in vitro* and *in vivo* behavior often offers great advantages. For instance, their chemistry and size can be precisely tuned in order to have the desired pharmacokinetic and pharmakodynamic characteristics, which furthers enhance transfection efficiency [116, 117]. Interestingly, dendrimers were also reported to act as drugs themselves. Supattapone *et al*. showed that branched polyamines, including PAMAM dendrimers stimulate the removal of prion proteins [137].

The number of studies using dendrimers as drug carriers [124, 138], and as contrast agents for magnetic resonance imaging has been increasing over the last years [129, 139, 140]. In fact, a few dendritic agents have already reached clinical studies: DEP™ docetaxel, a dendrimer with conjugated Docetaxel, is in Phase 1 clinical trials for the treatment of a wide range of solid tumors including breast, lung and prostate; VivaGel®, a G4 PLL-based dendrimer, which acts as antimicrobial agent in the treatment/prevention of a wide range of sexually transmitted diseases is in Phase 3 trials [141-146]; and Gadomer-17®, a PLL-based dendrimer bearing 24 DOTA-Gd chelates, is in Phase 2 trials as dendritic contrast agent for diagnosis in magnetic resonance imaging (MRI) [147, 148].

The use of dendrimers as NA carriers constitutes a less developed area when it comes to clinical applications. Nevertheless, thorough studies have already reported dendritic structures to be highly efficient NA delivery vectors. PAMAM is, by far, the most widely tested dendrimer for NA transfection, particularly DNA. The first report of a PAMAM dendrimer came up in 1993 [149]. Transfection efficiency was shown to increase with generation and appeared to be affected by the presence of fetal bovine serum. Even though high transfection rates were reported, cell viability decreased to 70 % and 35 % when treated with dendrimer alone or dendriplex, respectively. Cytotoxicity was then related with dendrimer size, concentration and dendrimer form (dendrimer alone or complexed with DNA). Nevertheless, this pioneering study proved that dendrimers are suitable candidates for NA delivery. Since then, a large

number of studies tried to understand the mechanisms behind dendrimer-mediated NA delivery. For instance, a study conducted by Smith *et al.* showed that high generation (G5-G7) PAMAM dendrimers were able to induce lipid mixing and leakage from negatively charged vesicles [97]. Their high internalization rates could be explained by these interactions with cellular membranes. Later, Sonawane *et al.* reported that PAMAM dendrimers have a high buffering capacity that slows down endosome acidification and further promotes endosomal swelling and disruption [150].

Several studies have also reported PAMAM modifications to reduce toxicity and to increase transfection efficiency, either by enhancing internalization or promoting cell-targeting. For instance, Saltzman *et al.* reported the synthesis of G5 PEG-conjugated PAMAM dendrimers and the efficient and non-toxic DNA delivery [127]. Even though transfection rates were 20-fold higher than the commercially available fractured PAMAM (Superfect™), the authors recognize that this system requires excessive amounts of dendrimer for an efficient DNA delivery. More recently, Tomás and co-workers, reported that the conjugation of alkyl chains of different lengths with G5 PAMAM dendrimers remarkably improved pDNA transfection and expression in mesenchymal stem cells with low levels of toxicity [151]. Interestingly, dendrimers functionalized with smaller alkyl chains were shown to promote higher levels of pDNA expression, which the authors hypothesized to be related with an enhanced pDNA dissociation due to weaker hydrophobic interactions and/or to a higher mobility of the complex across the cytoplasm [151]. On a different study, the same authors, reported the preparation of PAMAM dendrimers functionalized with peptides displaying high affinity for mesenchymal cells, which provided a receptor-mediated internalization and showed higher transfection rates than unmodified PAMAM dendrimers [152]. Similarly, PPI and PLL dendrimers have been also widely explored for DNA delivery [153-156].

Efforts are also being made to develop dendritic-based agents for siRNA delivery. However, the small size and lower charge density of siRNA leads to a less efficient interaction with cationic macromolecules when compared to pDNA, meaning that an efficient pDNA carrier may not be necessarily ideal for siRNA delivery. For instance, a family of triazine-terminated dendrimers, differing in their core flexibility, generation number, and surface functionality was developed by Merkel *et al.* and evaluated as pDNA and siRNA delivery agent [157]. The authors reported that the dendrimer with higher pDNA transfection efficiency was not able to induce siRNA-mediated gene-silencing [157-159].

Surface modification and functionalization has also been a common strategy to improve siRNA delivery. Qi and co-workers developed a G5 and G6 PAMAM dendrimer conjugated with PEG able to efficiently deliver siRNA, promoting gene silencing both in primary vascular smooth cells expressing GFP and GFP transgenic mice [160]. In a different approach, Minko and co-workers evaluated an internally quaternized G4-PAMAM-OH dendrimer as carrier for the targeted delivery of siRNA [161]. The neutral surface of these dendrimers elicited low cytotoxicity compared with the cationic counterparts. Interaction of the siRNA with the cationic charge inside the dendrimer led to the formation of a compact NP. Further functionalization with a synthetic analogue of LHRH peptide allowed the successful targeting of cancer cells and efficient siRNA uptake [161].

Despite the promising reports on both pDNA and siRNA delivery, furthers studies are needed to address the current limitations of dendrimer-based NA delivery, such as cytotoxicity.

1.3.2- Dendrimer toxicity

Dendrimer cytotoxicity is mainly associated to cell membrane disruption and subsequent non-apoptotic cell death. Numerous studies have thoroughly described the effect of dendrimer chemistry, size and charge on biological membranes integrity. However, recent reports suggest that, apart from membrane destabilization, toxicity may also arise from impaired oxidative metabolism, mitochondrial dysfunction and changes in endogenous gene expression that ultimately lead to apoptotic cell death.

As observed for other cationic macromolecules [111, 162], dendrimers with high positive densities display toxicity triggered by free amine groups at the periphery [114, 163]. Upon interaction with negatively charged biological membranes, cationic molecules induce hole formation and membrane thinning, which together cause loss of intracellular content and cell lysis [111, 164-167]. It is not totally clear how dendrimers interact with biological membranes, but several studies have already elucidated some of the mechanisms behind cellular internalization. A membrane bending model was proposed in 2000, describing the interactions between highly cationic dendrimers with anionic membranes through electrostatic forces [97]. The authors showed that high generation dendrimers were able to induce lipid mixing and leakage from the membranes, most likely owing to the high cationic profiles. A different mechanism was proposed by Mecke *et al.,* describing the formation of nanoholes (15-40 nm) upon dendrimer-membrane interaction [165].

As the generation increases, there is also an enhancement of the cationic density of the dendrimers. Thus it is reasonable to think that toxicity is proportional to dendrimer generation. In fact, several studies have already proven this hypothesis [168-170]. PAMAM cytotoxicity was shown to increase with generation as terminated G3-, G5- and G5-PAMAM dendrimers lead to a cell viability of 40, 20 and 10 %, respectively [168]. Likewise, Malik *et al.* demonstrated that several cationic dendrimers including PAMAM and PPI were, in general, cytotoxic and hemolytic in a generation- and concentration-dependent manner [169]. In contrast, their anionic forms did not show cytotoxicity nor hemolytic activity for the same concentrations. Functionalization of PPI dendrimers with either mannose or lactose improved cell viability by masking peripheral cationic amines [170]. Even though in these studies there are no experiments that point to the loss of membrane integrity, the authors hypothesize that cell viability decreases due to membrane disruption. The effect of dendrimer generation [169, 170], and surface properties on cytotoxicity have been extensively reviewed elsewhere [114, 163].

More recently, different studies have gone deeper in exploring different cellular parameters influenced by dendrimers. In 2005, Omidi and co-workers reported that PPI dendrimers could intrinsically modify endogenous gene expression [171]. Using microarray-based gene expression profiling, gene signature (altered genes) was found to be generation dependent, with G3-PPI dendrimer having a larger extent (10 %) of altered gene expression compared to G2 [171]. Gene expression modifications can severely impair different cellular pathways and mechanisms leading, for instance, to cell death by apoptosis or uncontrollable cell division.

Additionally, dendrimers were also shown mediate apoptosis through mitochondrial deregulation [172, 173]. G4- and G5-PAMAM dendrimers showed to induce down-regulation of several mitochondrial genes [172] and the disruption of the mitochondrial potential [173], respectively.

Thus, further studies on the impact of dendrimers in endogenous gene expression are needed to assess their safety and "off-target" effects or even to develop novel therapeutic targets and strategies.

Recently, several studies have highlighted the role of reactive oxygen species (ROS) in dendrimer-mediated cytotoxicity [174-177], For instance, PAMAM dendrimers induced ROS production and subsequently up-regulation of inflammatory mediators in a mouse macrophage cell line [175]. Mukherjee *et al*. later corroborated this study by demonstrating a generation-dependent ROS production, DNA damage and apoptotic activity in different mammalian cell lines treated with G4-,G5- and G6-PAMAM dendrimers [174].

Even though numerous *in vitro* studies have shown severe undesirable effects following administration of the widely used non-degradable PAMAM and PPI dendrimers, recent reports indicate that these effects cannot be always extrapolated to *in vivo* settings [178-180]. For instance, G6-PAMAM dendrimers did not induce any measurable hepatic damages in mice at 1, 5 or 10 mg/kg [179]. Similarly, unmodified G4-PPI bearing NH_2 at the periphery showed to be toxic by reducing food and water intake and body weight in mice [181]. Surface modification with maltotriose was shown to abolish toxicity effects, most likely due to charge masking [181].

However, other *in vivo* studies have provided conflicting results, evidencing toxicity profiles for the same dendrimers. For instance, G4-PAMAM dendrimers were shown to be toxic at a therapeutic dose of 7.1 mg/kg, as treated mice were more likely to die than non-treated animals [182]. Li and colleagues evidenced a major role of G3-PAMAM dendrimers bearing NH_2 groups in lung inflammation and injury in mice [183]. Upon 5 h of intratracheal administration of a dose of 50 mg/kg the survival rate dropped to 60 %, and 2 h post administration there was an 80 % change in lung elastance [183]. More recently, attention has been given to the formation of blood clots by dendrimers. NH_2-terminated G7-PAMAM dendrimers induced blood clot formation in a zebrafish model, possibly through electrostatic interactions between positive amine groups at the dendrimer's periphery and the negatively charged fibrinogen domains [184].

Cytotoxicity strongly depends on dendrimer characteristics, such as charge, size, and shape, that *in vivo* modulate biodistribution and pharmacokinetics. Additionally, it is clear that cytotoxicity is also related with dose concentration, time and type of administration and, hence, it is difficult to predict toxicity profiles based on *in vitro* experiments, given these barely mimic the complexity observed in biological systems.

Several studies have already reported that the conjugation of biocompatible molecules, such as PEG, decreased dendrimer cytoxicity by masking the positive charges that are thought to induce overall toxicity [185-187]. However, we believe that one key to solve the toxicity problems related with bioaccumulation would lay on the development of more biocompatible and biodegradable dendrimers. Their use could further prevent accumulation of synthetic materials by increasing cellular excretion and ultimately diminish some of the probable cytotoxic effects described above. Moreover, biodegradable dendrimers would allow the safe application of higher generations with enhanced multivalency, which were reported to have toxic profiles for non-degradable dendrimers.

Although the development of biodegradable dendrimers is still in its infancy, a few advantages have already been shown comparing with non-degradable dendrimers. For instance, Ye *et al* developed several biodegradable poly(ester) dendrimers for MRI that were

shown to promote lower tissue accumulation of toxic Gd(III) ions than non-biodegradable PAMAM dendrimers [188, 189].

1.3.3- Biodegradable dendrimers for gene delivery

Even though several groups have reported the success of dendrimers as NA delivery vehicles [151, 152, 190-197], the non-biodegradability of the used dendritic structures remains a drawback yet to be solved [117]. As previously mentioned, the ideal gene delivery vehicle should be biodegradable to prevent bioaccumulation and subsequent cytotoxicity [8, 117]. Moreover, biodegradability can contribute to the carrier's multivalency to decrease as a function of time, leading to a lower interaction with the transported NA, therefore promoting its release (Figure 1.9) [198, 199].

As in the case of linear polymers, the biodegradability of dendrimers can be achieved by inclusion in their structure of labile bonds that can be broken due to a specific biological activity or stimulus. For now, the majority of efforts have been focused on the development of dendritic architectures with hydrolyzable bonds, especially ester bonds. Polyester dendrimers represent an attractive class of nanomaterials for gene delivery and, in general, for biomedical applications, due to their good biocompatibility and the hydrolytic susceptibility of ester the bond to degrade under physiological conditions [200]. However, the development of these nanocarriers is challenging due to the lability of the ester bond during both the synthesis process and application [201, 202]. In fact, so far, not many strategies have been reported to synthesize biodegradable dendrimers and among those, only few suggest their suitability for application in the biomedical field [203]. Particularly for NA delivery very few examples on the application of bis-HMPA based dendritic compounds to encapsulate pDNA are found, which are described below.

Welsh *et al*. synthetized bis-HMPA based dendrons up to the 3rd generation (G3) with carbamate-linked spermine groups on the surface and a benzyl ester protecting group at the focal point that were shown to be degradable under physiological conditions [198]. The efficiency of pDNA binding increased in a generation dependent manner due to the enhanced multivalency [198]. In a slightly different approach, Barnard *et al*. replaced the benzyl ester protecting group by hydrophobic units (cholesterol and hydrocarbon chains) to promote their controlled self-assembly rendering a system with higher multivalency, what significantly enhanced the DNA binding. Furthermore, they modified the surface groups with N,N-di-(3-aminopropyl)-N-(methyl)amine (DAPMA), a triamine that showed lower cytoxicity than spermine [199]. Although the reported G2 dendrons could efficiently complex pDNA and undergo cellular internalization, a low transfection efficiency was observed. The authors hypothesized that at the lower endosomal pH, or when bound to DNA, the degradation of these dendrons becomes ineffective on the transfection time scale which could explain their poor transfection performance [199]. The same group further modified the cholesterol-functionalized G2 dendrons with small PEG chains (triethylene glycol and octaethylene glycol) [204]. PEG addition was found to affect size and zeta potential properties of the resulting dendriplexes and enhanced pDNA binding to different extents, with a higher binding affinity observed for the structures based on the longer PEG chain [204].

More recently, Barnard and co-workers have reported a different strategy targeting a better DNA release and dendron degradation [109]. They synthetized self-assembly disulfide-

linked dendron nanoparticles, where the cholesterol groups were attached to the DAPMA-terminated G2 bis-HMPA dendrons by an S-S linkage. In vitro, upon the addition of dithiothreitol, the nanoparticles undergo degradation due to the cleavage of the disulfide linkage, and consequently loss of self-assembled multivalent binding, triggering pDNA release [109]. Disulfide linkers are widely used for the design of non-viral gene delivery vectors due to their enhanced degradability in the reductive environment of the cytoplasm [12, 69].

Finally, a very recent report by Movellan *et al.* described the evaluation of an ionic G2 bis-HMPA dendrimer as pDNA vector. The dendrimer was functionalized with negatively charged hydrophilic chains of 2-[2-(2-methoxyethoxy)ethoxy]acetic in order to improve both solubility and biocompatibility [205]. G2 and G5 ionic PAMAM dendrimers equally functionalized were also used for comparison. Functionalization of bis-HMPA was shown to promote an enhancement of DNA encapsulation, yet smaller than PAMAM dendrimers. Nevertheless, preliminary transfection studies with pEGFP showed no significant GFP expression levels [205].

Even though there are already some reports on biodegradable dendritic structures for gene delivery, only a couple mention transfection studies [199, 205], with negligible efficiencies. This evidences that it is extremely difficult to go from synthesis to application. Moreover, to the best of our knowledge, there are no reports on biodegradable dendrimers for siRNA delivery.

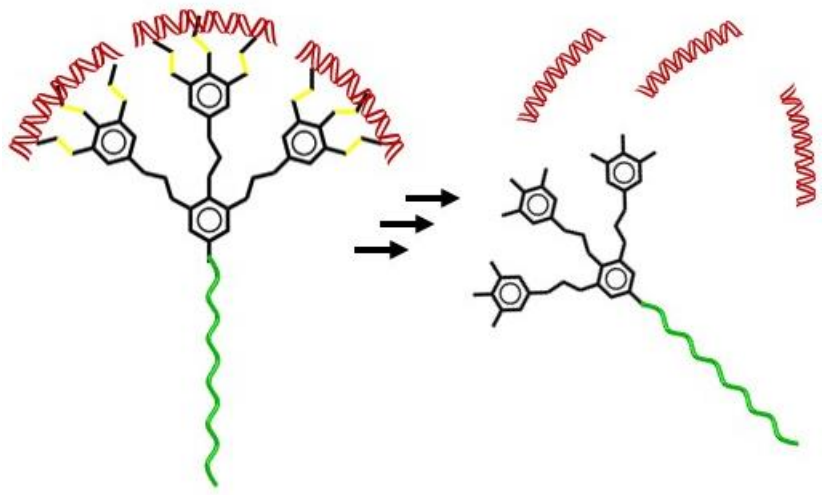


Figure 1.9 – Schematic representation of a biodegradable PEGylated dendron for siRNA delivery undergoing degradation. Black: dendritic backbone; Red: siRNA; Yellow: degradation points; Green: PEG.

Chapter 2 – Project

Here we report the amine functionalization of a new family of water-soluble, biocompatible and biodegradable PEG-bGATG dendritic block copolymers recently developed by our team, and their evaluation as siRNA delivery vectors.

The generation 2 of this new family of dendritic structures is composed by a non-biodegradable gallic acid-triethylene glycol (GATG) repetition unit monomer as core and three biodegradable GATG monomers (bGATG) forming the shell (Figure 2.1). The biodegradable trait is achieved through aliphatic ester bonds localized at the branches of the bGATG units, which are prone to degradation under physiological conditions. A 5 kDa PEG chain was attached to the focal point of the dendritic part to increase biocompatibility and circulation times. The presence of peripheral azides allows their functionalization by means of click chemistry with many different functional groups. The introduction of positively charged amine groups will allow to complex nucleic acids and, therefore, evaluate their performance as delivery vectors.

The aim of this work is to evaluate this new family of PEG-bGATG as vectors of siRNA. Thus, firstly, siRNA dendriplexes were produced and characterized in terms of physicochemical properties and, then, biological performance was assessed.

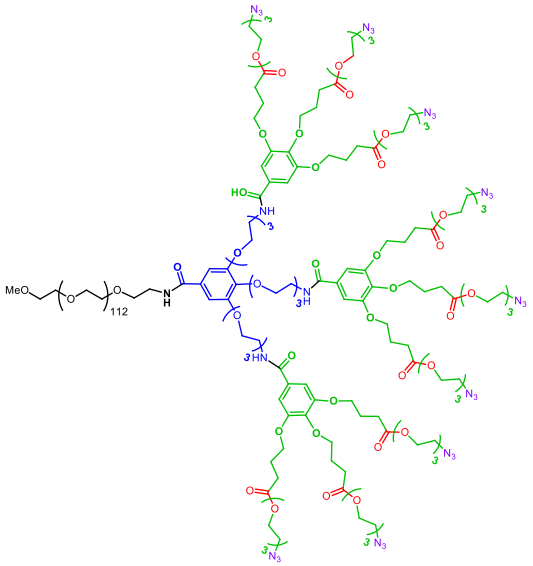


Figure 2.1 - Generation 2 (G2) of biodegradable PEG-bGATG dendritic block copolymers. Black: poly(ethylene glycol); Blue: non-degradable GATG monomer; Green: degradable bGATG monomer; Red: ester bonds; Violet: terminal azides.

2.1– Work plan

2.1.1 – Functionalization of PEG-GATG dendrimers with amine moieties by click chemistry

The first task consisted on the functionalization of the azide-terminated PEG-bGATG with a diamine (D) and a benzylmanine (Ar) by the Cu(I)-catalyzed Huisgen cycloaddition (CuAAC, click chemistry), yielding amine-terminated PEG-bGATG-D (bD) and PEG-bGATG-Ar (bAr) dendritic block copolymers, respectively. The cationic nature of these amine groups allowed the siRNA complexation through enhanced electrostatic interactions.

Their analogous, non-biodegradable PEG-GATG dendritic block copolymers were also functionalized with the same amine groups and used as controls throughout the study.

2.1.2– Preparation, characterization and optimization of PEG-GATG/siRNA dendriplexes

Dendriplexes were prepared by mixing dendrimer volumes with siRNA obtaining different N/P ratios (N/P ratio describes the ratio of moles of the amine groups of the dendrimer to those of the phosphate ones of siRNA). The resulting dendriplexes were characterized and optimized in terms of size, polydispersity, charge and morphology. Furthermore, complexation efficiency and stability were measured by nucleic-acid binding dye exclusion assay and gel electrophoresis.

2.1.3– Biological performance

Dendrimer and dendriplex cytoxicity were measured by the resazurin reduction assay. Cytotoxicity was further evaluated by evaluation of the hemolytic activity of the dendritic structures produced.

The *in vitro* performance of the developed dendriplexes was evaluated regarding their cellular internalization capacity using both Fluorescence-Activated Cell Sorting (FACS) and imaging flow cytometry. Finally, silencing efficiency was determined by measuring Luciferase activity, upon the transfection of U2OS cells stably expressing the fusion protein EGFP-Luciferase.

Chapter 3- Materials and methods

The following table includes the reagents used throughout the experimental procedures and the respective suppliers.

Table 3.1 - Materials and Suppliers.

Reagent/Material	Supplier
PEG-GATG-N_3/PEG-bGATG-N_3	Synthesized by our team
N-2-propyn-1-yl-1,3-propanadiamine.2HCl (Diamine), 95 %	Amatek Chemical Co Limited
4-ethynyl-benzenemethanamine.HCl (Benzylamine), 95 %	Amatek Chemical Co Limited
Sodium Ascorbate, 98 %	Sigma-Aldrich
Copper(II) Sulfate Pentahydrate, 95 %	Riedel-de-Haen
DMF, synthesis grade	Sigma-Aldrich
Amicon® Stirred Cells	Merck Millipore
Ultracel® Ultrafiltration Discs	Millipore Iberica, S. A.
Nuclease Free Water	Qiagen
SiDNA/siRNA	Integrated DNA Technologies
0.6 ml Snaplock tubes	Axygen
Chloroquine C6628	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Gibco
Trypsin T-0646	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco
Opti-MEM	Gibco
Gentamycin	Gibco
SYBR Gold Nucleic Acid Stain	Life Technologies
Luciferase Assay System	Promega
BCA Protein Assay Kit	Pierce
Hoechst 33342	Life Technologies
Histopaque-1077	Sigma-Aldrich
96-well Polystyrene Round Bottom Plates	Sigma-Aldrich
BCA Protein Kit	Thermo Scientific

3.1 – Methods

3.1.1 – Functionalization of PEG-GATG dendrimers (G2) with positively charged amine moieties by click chemistry

PEG-dendritic block copolymers (PEG-bGATG-N_3 and PEG-GATG-N_3) were dissolved in DMF/H_2O (1:1) to give a 0.1 M final concentration of terminal azides. Then, alkynylamines (N-2-propyn-1-yl-1,3-propanadiamine.2HCl and 4-ethynyl-benzenemethanamine.HCl) (200 mol % per terminal N3) and aqueous solutions of $CuSO_4.5H_2O$ 0.1 M (5 mol % per N3) and sodium ascorbate 0.1 M (25 mol % per N3) were added. The resulting solution was stirred at room temperature for 24 h, and the product was isolated by ultrafiltration ($EDTA_{aq}$ 0.1 M pH 6, $NaCl_{aq}$ 0.6 M, H_2O) of the concentrated reaction mixture (Ultracel® 1,000 MWCO). The corresponding amine-terminated compounds were obtained in excellent yields: 100, 100, 94 and 98 % for bD, bAr, nD and nAr, respectively.

3.1.2 – Dendriplex preparation

Dendrimer/siRNA complexes were prepared at different N/P ratios (where N = number of primary amines in the conjugate; P = number of phosphate groups in the siRNA backbone) ranging from 20 to 160 by adding siRNA (20uM) to different volumes of dendrimer solution (6 mg/mL) in Nuclease-Free (NF) water. Then, the resulting dendriplex solutions were vortexed for 10s and incubated for 30 min at RT prior to experiments.

For the experiments where no biological activity (silencing) was required, we used annealed sense and antisense DNA strands mimicking siRNA, as DNA oligos are easier to synthesize and obtain in high yields and purity.

3.1.3 – Size and zeta potential measurements

Size, dispersion (PDI) and zeta potential (ZP) were measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments, UK) following the manufacturer instructions. Size and PDI were determined at RT with a scattering angle of 173° using a ZEN0040 cell in the automatic mode, and the mean hydrodynamic diameters were determined by cumulative analysis (Z-average mean). Zeta potential measurements were performed using the same instrument and the same conditions. For size and PDI measurements dendriplexes were prepared in a final volume of 80 uL. For ZP measurements dendriplexes were prepared in a final volume of 250 uL and diluted to 750 uL in milli Q water previously to the measurements using a capillary cell (DTS1070). The Smoluchowski model was applied for zeta potential determination, and cumulant analysis was used for mean particle size determination.

The presented data are expressed as the mean ± SD of three independent sample measurements. The software used was DTS Nano version 7.1, supplied by the manufacturer (Malvern Instruments, UK).

3.1.4 – Transmission electron microscopy

Dendriplexes were prepared as previously described at N/P ratios of 80 and 160. Samples were mounted on a 200-mesh Ni grid with Formvar and carbon supporting film (not glow discharged) and stained with 2% uranyl acetate (UA) solution. Excess stain was removed with filter paper, and the grid was dried prior to imaging. Samples were imaged using a Jeol JEM 1400 operated at 80 kV. Images were processed using ImageJ software (NIH, USA).

3.1.5 – Polyacrylamide gel electrophoresis shift assay

Polyacrylamide gels (with 4% stacking and 15% resolving gel) were prepared in Tris/Borate/EDTA buffer. Dendriplex solutions were prepared at different N/P ratios as previously described with the difference that "siDNA" (where the RNA nucleotides were substituted by DNA) was used instead of siRNA. The amount of dendriplex corresponding to 12 pmol of siDNA was mixed with 6 uL of loading buffer and subjected to gel electrophoresis at 100 V. Dendriplex/siDNA binding was shown by a lack of migration of the siDNA in the electrophoretic field.

3.1.6 – SybrGold exclusion assay

siDNA condensation efficiency was assessed by measuring the percentage of *siDNA* complexed with the developed dendrimers, using the SYBRGold® exclusion assay. For that, SYBRGold® was used to measure free *siDNA* (not complexed into nanoparticles) after nanoparticle preparation

Dendriplex/*siDNA* nanoparticles were prepared as previously described and then incubated in NF water (Qiagen) for 10 minutes at RT in a 96-well black plate with 2 µL of a 1:100 SYBRGold (Invitrogen) solution (in TAE buffer) (final volume of 200 µL). After incubation, fluorescence was measured (λ_{exc} = 485 nm, λ_{em} = 540 nm) using a multimode micro-plate reader (SynergyMx, Biotek). Results are given as percentage of complexation, where 100% represents complete siRNA complexation.

The presented data are expressed as mean ± SD of three independent sample measurements.

3.1.7 – Nuclease protection assay

Dendriplexes with a N/P ratio of 160 were prepared as previously described and incubated with 0,1 U DNase I per 0,2 ug of siDNA for 5 and 15 minutes at RT. For DNase inactivation samples were treated with EDTA (0.05 M final concentration), heated up to 65º C for 10 min and further stabilized for 30 min at RT. Mixtures were treated with sodium dodecyl sulfate (SDS) to a final concentration of 0.1 % and incubated for another 30 min.

As control, 1 pmol of siDNA was diluted in NF water (final volume 10 uL), mixed with LB and further loaded into a polyacrylamide gel (10 %). Both naked siDNA and untreated dendriplexes were used as controls. Free wells were loaded with equivalent concentrations of salts, SDS and EDTA to allow a uniform band migration.

3.1.8 – Cell culture

The osteosarcoma cell line U2OS were cultured in DMEM media supplemented with 10% (v/v) FBS and 40 ug/mL gentamicin.

3.1.9 – Cytotoxicity studies

Cell viability was evaluated as a function of the dendrimer and dendriplex concentration and N/P ratio, respectively. Cells were seeded in 96-well plates at a density of 3.75×10^4 cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 70-80% confluence. At the time of transfection, the medium was replaced for non-supplemented DMEM.

24 h post-transfection, the medium was replaced for fresh media containing 10 % FBS and 10 % resazurin and incubated for another 3 h. Fluorescence (λex = 530 nm, λem = 590 nm) was measured in a multimode micro-plate reader (SynergyMx Plate Reader, Biotek). The viability of cells exposed to dendrimer was expressed as a percentage of the viability of non-treated cells. The presented data are expressed as the mean ± SD of three independent sample measurements.

3.1.10 – Lethal concentration 50 (LC50) studies

Cell viability was evaluated as a function of the dendrimer concentration. Cells were seeded in 96-well plates at a density of 1.875×10^4 cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 50% confluence. At the time of transfection, the medium was replaced for non-supplemented DMEM.

24 h post-transfection, the medium was replaced for fresh media containing 10% FBS and 10% resazurin and incubated for another 3 h. Fluorescence (λex = 530 nm, λem = 590 nm) was measured in a micro-plate reader spectrophotometer (SynergyMx Plate Reader, Biotek). The viability of cells exposed to dendrimer was expressed as a percentage of the viability of non-treated cells.

The presented data are expressed as the mean ± SD of three independent sample measurements.

3.1.11 – Hemolytic activity

Red blood cells (RBCs) were isolated from buffy coats (obtained from Immunohemotherapy Service, Hospital S. João, Porto, Portugal), as described previously.[206]

Briefly, RBCs were centrifuged over a density gradient with Histopaque-1077 according to the manufacturer's instructions. After removal of the plasma upper layer, the lower layer containing RBC was washed three times in PBS. The purified RBCs were diluted to a concentration of 2 x 10^8 cells/ml and 150 µL of RBCs were placed in round bottom 96-well polypropylene microtiter plates. Then, 150 µL dendrimer/dendriplex solutions were added to the wells and incubated for 1h at 37 °C. Afterwards, the plates were centrifuged at 4000 rpm for 15 min, and the supernatant transferred (100 µL) from each well to black polypropylene 96-wells microtiter plates for absorbance reading. Absorbance was read at 380, 415, and 450 nm using a micro-plate reader spectrophotometer (SynergyMx Plate Reader, Biotek). The amount of Hemoglobin (Hb) is calculated as follows: Hb value of sample (mg/dl) = [[2 × A415 − (A380 + A450)] × 1000] / (E), where A415, A380, A450 are the absorbance values at 415, 380, and 450 nm, respectively. A415 is the Soret band absorption of Hb and A380, A450 are correction factors of uroporphyrin whose absorption falls under the same wavelength range. E is the molar absorptivity of oxyhemoglobin at 415 nm, which is 79.46. The hemolytic potential of both dendrimers and dendriplexes was calculated as: Hemolysis (%) = Hb value of sample / Total Hb value × 100, where total Hb value corresponds to 100 % hemolysis with 1% triton X-100 . Dendrimer concentrations tested were 0.25, 0.5, 0.75, 1 and 1.5 mg/mL[1]. PBS 1x and 2% Triton X-100 (Sigma Aldrich) in PBS were used as negative and positive lysis controls, respectively.

The presented data are expressed as the mean ± SD of three independent sample measurements.

3.1.12 – Flow-cytometry

Cells were seeded in 24-well plates at a density of 2.6 ×10^4 cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 70-80% confluence prior to transfection. At the time of transfection, the medium was replaced with non-supplemented DMEM. Dendriplexes were prepared with Cy-5 labeled siRNA as described above. Cells were then transfected using 50 uL dendriplexes in a final volume of 300 uL (siRNA concentration of 0.1 pmol/ul). After 24 h incubation, cells were rinsed twice with 1x PBS, trypsinized, centrifuged, resuspended in 1x PBS 2% FBS and analyzed by FACS (FACSCalibur, BD Biosciences). Non-treated cells and cells transfected with Lipofectamine 2000 (Life Technologies) were used as negative and positive controls, respectively. Data was analyzed using FlowJo software (version 8.3.7).

3.1.13 – Confocal microscopy

Cells were seeded in 8-well uncoated µ-Slide (Ibidi) at a cell density of 2 x 10^4 cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 50-60% confluence prior to transfection. At the time of transfection, the medium was replaced for non-supplemented DMEM. PEG-bGATG-Ar/siDNA and PEG-bGATG-D/siDNA dendriplexes with N/P ratios of 160 were prepared using Cy-5 labeled siDNA. . Cells were then transfected using 50 ul dendriplexes in a final volume of 300 ul (siRNA concentration of 0.1 pmol/ul). After 24 h, transfected cells were washed three times with PBS and incubated 10

minutes (RT) with a 1:20000 diluted solution of Hoechst 33342 (10 mg/ml) for nuclear staining. Cells were then washed with PBS and Opti-MEM (no phenol-Red) was added to cells prior to microscopy. Cells were imaged with a Leica TCS SP2 AOBS confocal microscope.

Three-dimensional z-stacks were captured and processed using ImageJ software (NIH, USA)

3.1.14 – Imaging Flow Cytometry

Cells were seeded in 6-well plates at a density of 3.2 x 10^4 cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 70-80% confluence prior to transfection. At the time of transfection, the medium was replaced by non-supplemented DMEM. PEG-bGATG-Ar/siRNA dendriplexes with a N/P ratio of 160 were prepared using Cy-5 labeled siRNA. Cells were then transfected with a final volume of 350 ul (final siRNA concentration 0.1 pmol/u). Lipofectamine 2000/siRNA complexes were used as positive control, following the manufacturer's instructions. 24 h after incubation, cells were rinsed once with PBS 1X and trypsinized. Cells were then transferred to Eppendorfs and centrifuged for 5 min at 1200 rpm at 4ºC. After washing with PBS 1X, cells were centrifuged (5 min at 1200 rpm at 4ºC), then cells were fixed with 4 % paraformaldehyde (PFA) for 15 minutes. After the fixation procedure, cells were washed twice with PBS 1X.

Cell images were acquired using an imaging flow cytometer (ImageStream®, Amnis, EDM Millipore, Darmstadt, Germany), at 40x magnification.

A 488 nm wavelength laser was used to excite Cy-5 labeled *siDNA*. The fluorescence images were collected using the 660-745 nm spectral detection channel. Cell images were analyzed using IDEAS image-analysis software (Amnis Corporation, EDM Millipore).

Every cell was divided into 2 regions – external (membrane) and internal (cytoplasm). The external region was determined by dilating the mask by the membrane thickness and removing the internal region, which gives the mask from the cell membrane. The internal region was defined by the mask of the whole cell minus the cell membrane mask.

A mask was then attributed to the intensity of the Cy5 channel. The intensity mask was merged with the cytoplasm mask and then the values for fluorescence intensity were plotted in a graphic and statistically analysed.

IDEAS software allows the quantification of the number of nanoparticle-loaded vesicles (NLV). Thus, images were also acquired using the Extended Depth of Field (EDF) filter, which uses a combination of specialized optics and image algorithms to project all structures within the cell into a single plane of focus. A cell is imaged several times in different focus planes in the z axis that then are all merged into a single, entirely focused composite image. To determine the number of vesicle-loaded nanoparticles, masks were created which identify the fluorescence spots. The number of individual vesicles in a cell was enumerated using the Spot Count feature from the software, and plotted in frequency histograms. Three regions (low, medium and high spot count) were defined based on the worst performing dendrimer (PEG-GATG-D). The region for medium spot count is defined as mean spot count for PEG-GATG-D (3.5) ± its corresponding standard deviation (2). The low spot count region is below 3.5 – 2 and the high spot region is above 3.5 + 2.

3.1.15 – Silencing studies

Cells were seeded in 24-well plates at a density of 2.6 $\times 10^4$ cells/cm^2 and incubated for 24 h in supplemented DMEM medium at 37 °C, 5% CO2, and grown to reach 70-80% confluence prior to transfection. At the time of transfection, the medium was replaced for non-supplemented DMEM. Dendriplexes with N/P ratios of 160 were prepared as previously described. Cells were then transfected using 50 ul dendriplexes in a final volume of 300 ul (siRNA concentration of 0.1 pmol/ul). After 24h incubation, cells were treated with chloroquine (100 nM) and further incubated for 4 h. Then, the medium was replaced for fresh supplemented DMEM and incubated another 44 h. Cells were rinsed twice with PBS and then incubated on ice with 0,15 % Triton X-100 HKR buffer for 5 min. Cell lysates were centrifuged at 400 xg for 5 minutes and supernatants were further analyzed for luciferase activity with Promega's luciferase assay reagent following the supplier's instructions. Light emission was measured using a micro-plate reader (SynergyMx, Biotek). Protein concentration in cell lysates was measured using the BCA Protein Assay Kit. Luciferase activity of treated cells was expressed as a percentage of luciferase activity measured for non-treated cells.

The presented data are expressed as the mean ± SD of four (n=4) independent sample measurements.

3.1.16 – Statistical analysis

Data are given as mean ± SD, with *n* denoting the number of repeats. Significant differences were examined using one-way ANOVA. Tukey's multiple comparison test was further employed after one-way ANOVA for samples were homogeneity of variances was observed. Games-Howell multiple comparison test was employed after Welch ANOVA for samples violating homogeneity of variances. Independent t-tests were also carried on for some experiments. A *p* value of <0.05 was considered statistically significant in all studies. Statistical analyses were performed using IBM SPSS Statistics 21 software.

Chapter 4 – Results and discussion

4.1 – Functionalization of PEG-GATG dendrimers with positively charged amine moieties

It is widely found throughout the literature that positively charged nanoparticles promote higher and stronger electrostatic interactions with negatively charged nucleic acids, favoring a more efficient ionic condensation [11, 123]. In a gene therapy setting, positive charges are equally important because it facilitates cell adsorption and mediates efficient endosomal uptake into cells [11, 123].

Here, two different amine groups were chosen for the surface modification of the dendritic structures: a) a diamine group (N-2-propyn-1-yl-1,3-propanadiamine) bearing two positive charges was used to increase the multivalency of the dendrimer without increasing its generation; b) a benzylamine group (4-ethynyl-benzenemethanamine) was used to evaluate the effect of hydrophobic interactions in the complexation process.

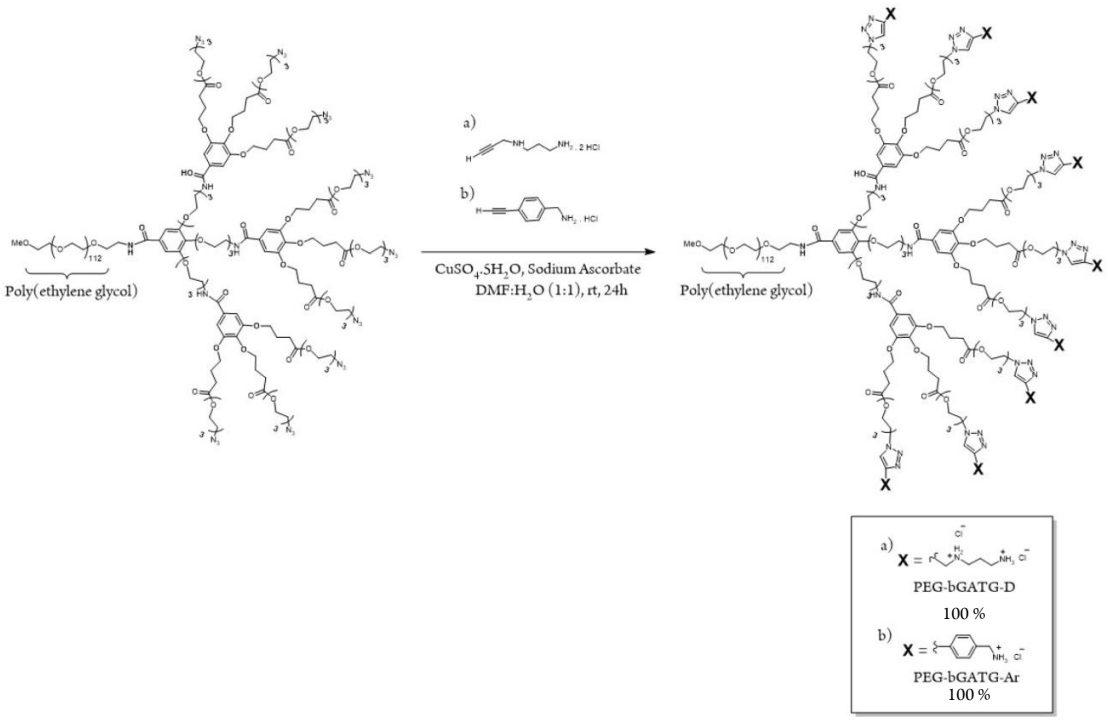


Figure 4.1 - Functionalization of the PEG-bGATG surface by click chemistry with a) diamine (PEG-bGATG-D, bD), and b) benzylamine (PEG-bGATG-Ar, bAr).

Surface modification of the PEG-bGATG dendritic block copolymers was carried out using the Cu(I)-catalyzed Huisgen cycloaddition following standard conditions: CuSO4 (5 mol % per azide) as source of copper and sodium ascorbate (25 mol % per azide) as reducing agent, under aqueous conditions (DMF/H2O, 1:1) at room temperature for 24h (Figure 4.1).

The resulting amine-terminated biodegradable PEG-bGATG diamine derivative (bD) and benzylamine derivative (bAr), incorporating protonated amine groups, were purified by ultrafiltration and obtained in excellent yields: 100 % (Figure 4.1).

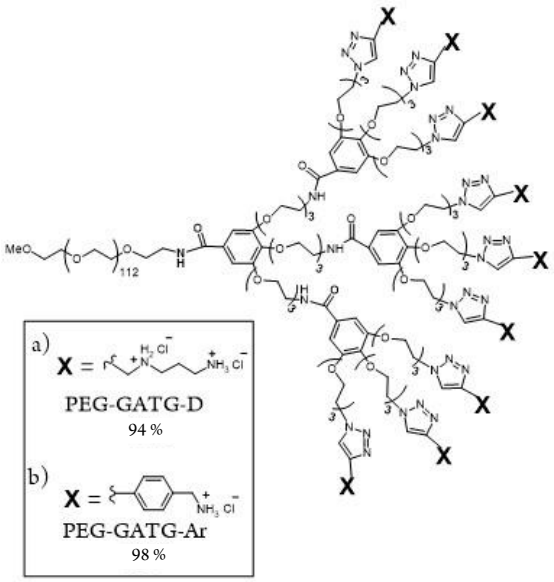


Figure 4.2 - Structures of a) propanediamine derivative (PEG-GATG-D, nD), and b) benzylamine derivative (PEG-GATG-Ar, nAr) of non-biodegradable PEG-GATG.

The successful incorporation of the amine moieties at the surface of the PEG-bGATG dendrimers was confirmed by ^{1}H NMR (D_2O) and FTIR spectroscopy (KBr technique) (data not shown).

Non-biodegradable PEG-GATG dendritic block copolymers were equally functionalized with both amine moieties and used as control throughout the study to prove the working hypothesis that biodegradable systems outperform non-biodegradable ones (Figure 4.2).

4.2 – Dendriplex characterization

The association of the amine-terminated biodegradable (bD and bAr) and the non-biodegradable (nD and nAr) PEG-GATG dendrimers with anti-GPF *siDNA*/siRNA was studied and the resulting dendriplexes evaluated regarding their physicochemical properties.

4.2.1– Complexation efficiency

The complexation process between dendrimers and nucleic acids does not differ from other cationic molecules, and it mostly driven by electrostatic interactions.[123]

The potential of every nanomaterial to bind and compact nucleic acids is one of the key factors for an efficient gene delivery [11, 123]. Thus, the ability of the developed PEG-dendrimers to complex anti-GFP *siDNA* was first evaluated by polyacrylamide gel retardation assay (Figure 4.3a). Gel retardation assay is a widely used qualitative technique to assess protein/nucleic acid interactions. Here, we expected that as the interactions between dendrimers and the nucleic acid increase, a lower electrophoretic mobility of the nucleic acid across the gel is observed [207].

As shown in Figure 4.3a, the amount of free siDNA – siDNA able to migrate – decreases with the N/P, which is expected since we are increasing the amount of PEG-dendrimer per siDNA molecule and hence favoring the complexation process. Additionally, diamine-terminated dendrimers, either biodegradable or non-biodegradable, seem to be more efficient, namely at lower N/P's. This is likely due to the presence of a higher density of positive charges that strengthen electrostatic interactions.

Between non-biodegradable and biodegradable PEG-dendrimers with the same terminal group (nD vs bD and nAr vs bAr), the latter show a higher level of *siDNA* retention when comparing with naked *siDNA*. Contrary to the non-degradable PEG-GATGs, the presence of more hydrophobic branches in the backbone of the biodegradable PEG-bGATG dendrimers can provide a different *siDNA* packaging, and hence positively contribute to the complexation efficiency.

The condensation efficiency was also quantitatively measured by an nucleic acid-dye binding assay using SybrGold, which is a very sensitive dye that binds to free nucleic acids. As Figure 4.3b shows the amount of condensed *siDNA* is ≥ 65 % for every PEG-dendrimer and N/P studied and ≥ 85 % for N/P's ≥ 80. Even though the amount of condensed *siDNA* seems to increase with the N/P there are no statistical significant differences.

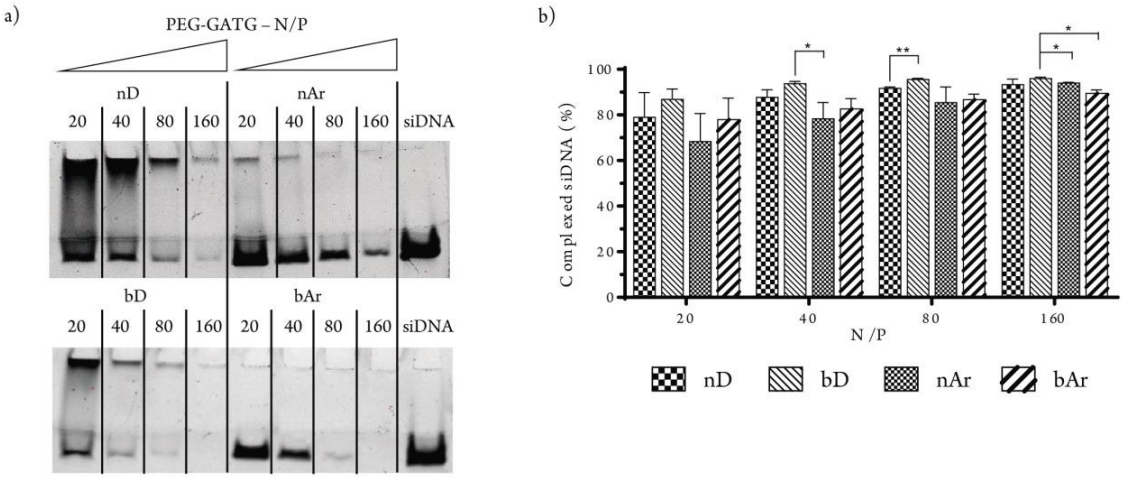


Figure 4.3 – Complexation efficiency a) Polyacrylamide gel retention assay of the siDNA dendriplexes from: non-biodegradable PEG-GATG (nD and nAr) and biodegradable PEG-bGATG (bD and bAr). (N/P ratios are indicated above each column. In both panels the last column corresponds to naked siDNA). b) SybrGold exclusion assay at room temperature. Results are expressed as mean ± SD of three independent measurements (n=3). One-way ANOVA tests were used for statistical analysis. Significant differences: *$p < 0.05$, **$p < 0.01$. Significant differences between N/P's: nAr 20 vs. nAr 160 ($p < 0.05$).

The SyrbrGold assay shows that diamine-terminated PEG-dendrimers are more efficient in siDNA complexation, which is in line with the results for the gel retardation assay. Additionally, this experiment proves that the PEG-bGATG-D is the best performing PEG-

dendrimer when it comes to *siDNA* packaging, having close to 90 % of complexed siDNA at the lowest studied N/P.

4.2.2- Dendriplex size, surface charge and morphology

A fundamental difference is observed when comparing pDNA complexes with siRNA complexes: siRNA molecules are smaller, and have a lower charge density, which promotes a less strong interaction with cationic molecules. This often leads to incomplete encapsulation and formation of large complexes, which constitutes a major challenge for non-viral siRNA delivery [123, 191].

Thus, the size (hydrodynamic diameter) and polydispersity indexes (PdI's) of the produced dendriplexes were measured by Dynamic Light Scattering (DLS) (Figure 4.4a and 4.4b). A particle size in the nanometer scale and narrow PdI's were found for every dendriplex at each N/P ratio, which can be considered suitable for gene delivery/cellular uptake setting [86].

Although there was no observable influence of the degradability trait on size and PdI, the nature of the terminal group was shown to be determinant for particle size. Hydrodynamic diameters ranging from 130 to 160 nm and from 165 to 195 nm were obtained for diamine- and benzylamine-terminated dendriplexes, respectively (Figure 4.4a). Regarding PdI, which measures the homogeneity of a population, benzylamine-terminated dendriplexes were shown to have more defined sizes with values around 0.3, while diamine-terminated particles had values closer to 0.4 (Figure 4.4b). Therefore, the additional hydrophobic interactions that arise between the benzylamine and the *siDNA* appear to lead to more homogeneous dendriplexes.

Even though particle size seems to increase with N/P ratio, no significant differences were found except for PEG-bGATG-D (40 vs. 160).

Moreover, the morphology of the dendriplexes was shown to be globular and spherical for every PEG-dendrimer, independently of their degradability or terminal group, as shown by TEM images (Figure 4.4).

For every case, sizes correlated with those obtained by DLS (Figure 4.4a). Any difference can be explained by protocol variations between both techniques: DLS measurements are carried out in solution, therefore, the hydrodynamic diameter is measured; while by TEM samples are dried up before analysis, so a smaller diameter can be obtained.

Previous reports on pDNA complexation with non-biodegradable PEG-GATG-NH_2 (G2) and other polycationic systems with high PEG loadings showed less packed and more extended morphologies [77, 208-210]. Therefore, these results show that functionalization of PEG-GATG with these amine moieties (diamine and benzylamine) is extremely important for the encapsulation of nucleic acids in well-defined structures.

The surface charge of dendriplexes was measured by laser Doppler electrophoresis (Figure 4.4c). For every tested condition, dendriplexes were shown to have positive global net charge ≥ 8 mV, which, as stated previously, is extremely important for an efficient cellular internalization. Again, these results showed that the main differences are observed independently of bio- or non-biodegradable PEG-GATG, with higher surface charge values obtained for the diamine-terminated structures (values between 17 and 23 mV for N/ P ≥ 40).

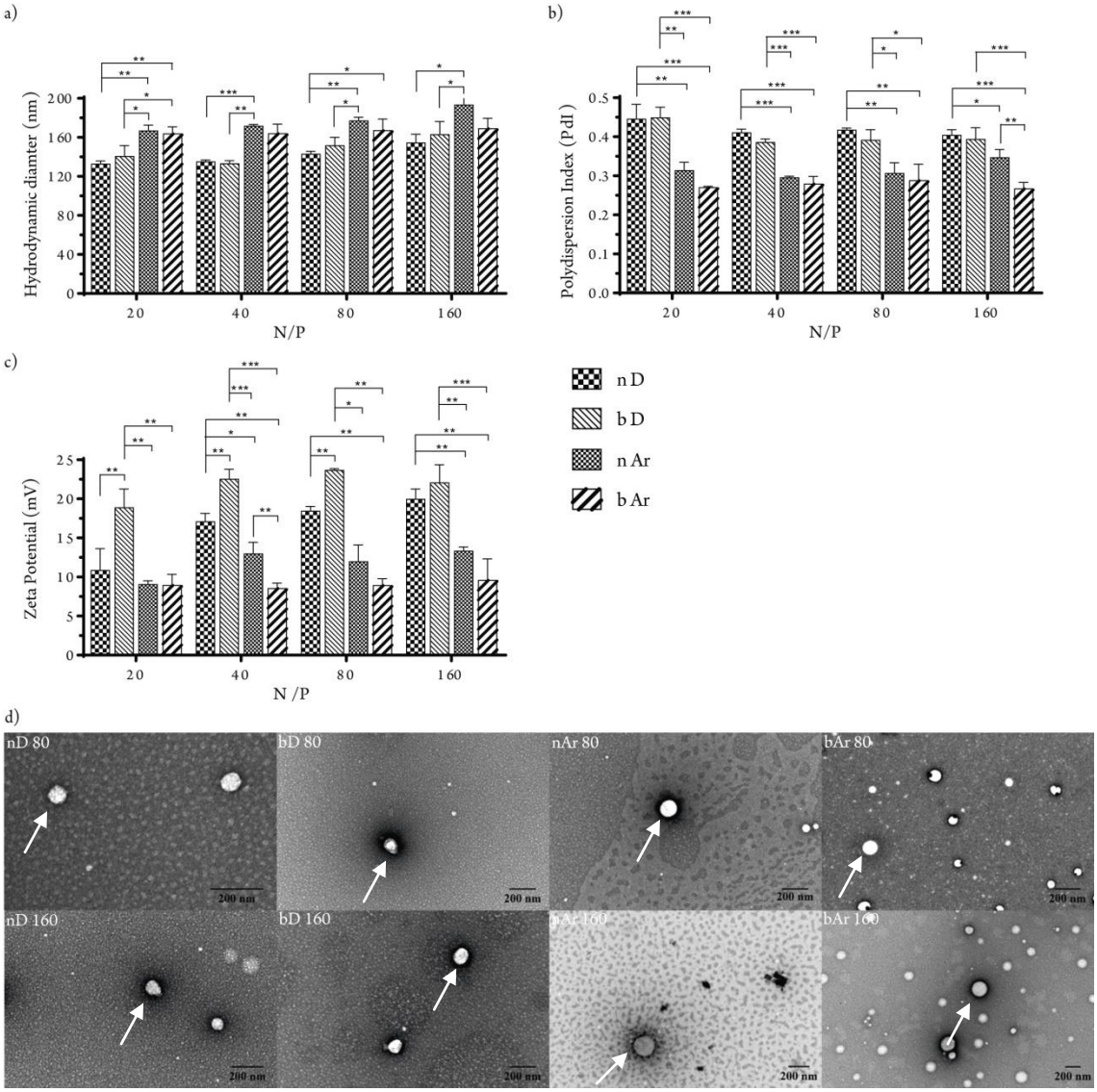


Figure. 4.4 - Size, surface charge and morphology for biodegradable and non-biodegradable PEG-GATG/*siDNA* dendriplexes. a) Size distribution of siDNA dendriplexes measured by DLS at different N/P ratios (n=3, mean ± SD). Significant differences between N/P's: bD 40 vs bD 160 ($p < 0.05$). b) PdI's for PEG-GATG/siDNA dendriplexes measured by DLS at different N/P ratios (n=3, mean ± SD). No significant differences between N/P's. c) Potential zeta values for all developed dendriplexes at different N/P ratios (n=3, mean ± SD).. Significant differences between N/P's: bD 20 vs bD 80 ($p < 0.05$), nD 20 vs nD 40/80/160 ($p < 0.01$), nAr 20 vs nAr 160 ($p < 0.01$). d) TEM images for siDNA dendriplexes (arrows) at N/P 80 and 160: nD, nAr, bD, and bAr. One-way ANOVA tests were used for statistical analysis. Significant differences: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

On the other hand, lower values between 8 and 13 mV, were obtained for benzylamine-terminated PEG-dendrimers. These results were expected due to the higher density of positive charges for the diamine group. Differences between PEG-dendrimers with the same terminal group can be owed to different packaging of the nanoparticles.

4.3 - Biological performance

The developed biodegradable and non-biodegradable PEG-GATG dendritic compounds were further evaluated regarding their ability to protect nucleic acids from endonuclease degradation, toxicity, hemolytic activity, cellular uptake and transfection efficiency.

4.3.1 – Protection against endonucleases

The successful development of a delivery vector requires the NA to remain intact and active until it reaches the target cells. Therefore, the vector has to protect it from enzymatic degradation both in serum and extracellular milieu [8, 70].

Thus, to evaluate the ability of all the developed PEG-GATG dendrimers to protect *siDNA*, dendriplexes at N/P 160 were prepared and incubated for different time periods (5, 15, 30 and 60 min) with DNase I. After incubation, the intact *siDNA* was displaced from the dendriplexes and samples were analyzed by polyacrylamide gel electrophoresis (Figure 4.5).

While naked *siDNA* (control), fully accessible to enzyme activity, was completely degraded within 5 min of incubation, protection of *siDNA* to different degrees was observed for every dendriplex (Figure 4.5). As expected, a higher *siDNA* protection was observed at lower time points (5 and 15 min). However, even at 30 and 60 min a significant level of *siDNA* protection is observed, namely for the benzylamine-terminated PEG-bGATG (bAr). Again, this result validates the importance of the additional hydrophobic contribution provided by the aromatic benzylamine moieties.

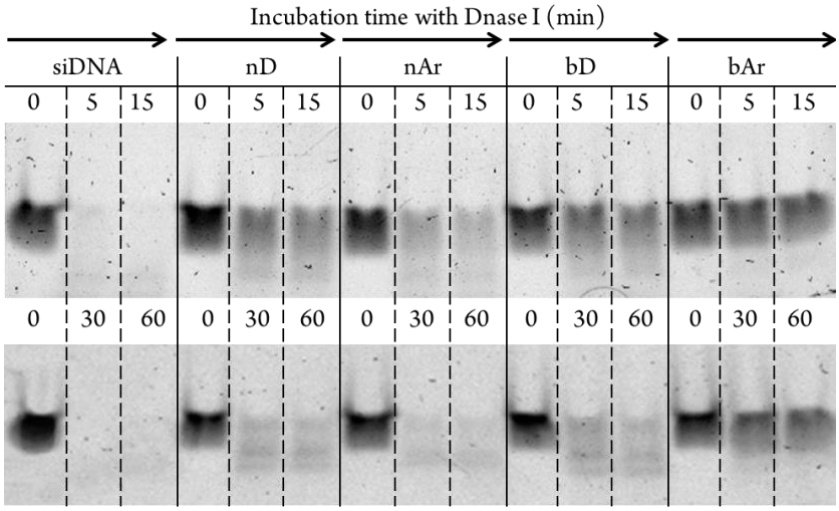


Figure 4.5 - Protection against endonucleases by polyacrylamide gel electrophoresis after 0, 5, 15, 30 and 60 min incubation with DNase I. From left to right: Naked siDNA, PEG-GATG-D, PEG-GATG-Ar, PEG-bGATG-D and PEG-bGATG-Ar.

Previous studies showed that the non-biodegradable G3 of PEG-GATG were not able to protect the associated pDNA from degradation during long time periods (> 5 min) [193]. Once more, our results reveal the importance of surface functionalization with these positively charged amine moieties, which contribute to more stable complex formation.

4.3.2 - Cytotoxicity

The main drawback hampering a faster transition of dendrimers from simple testing molecules to clinically acceptable therapeutics is their associated and thoroughly reported *in vitro* cytotoxicity. As already mentioned, the toxicity of some of the most commonly used dendrimers such as PAMAM and PPI is attributed to the high density of cationic surface groups. These are thought to interact with the negatively charged cellular membrane and

promote cell lysis [111, 164-168]. Many studies have confirmed that surface modifications like PEGylation reduce toxicity through partial shielding of the positive charges [112, 185-187].

For instance, Kim *et al*. showed that a G5 PAMAM-PEG-PAMAM triblock copolymer did not induced any decrease in 293 cells' viability at 150 μg/ml, while G4 PAMAM and PEI displayed cytotoxicity values around 80 and 20 %, respectively [185]. Likewise, Tang and co-workers reported that both G5 and G6 PEG-PAMAM dendrimers, when incubated with Cos-7 cells, decreased the toxicity in more than 30 % at 0.256 mg/ml when comparing with non-PEGylated PAMAM dendrimers [160].

To the best of our knowledge there are only two works reporting cytotoxicity studies of biodegradable dendrimers as nucleic acid vectors. Barnard *et al*. tested concentrations up to 60 ug/ml for the bis-HMPA dendron modified with cholesterol and hydrocarbon chains [199]. While cholesterol-modified dendrons did not alter cell viability, dendrons bearing hydrocarbon chains were shown to be more cytotoxic than PEI for HEK293 cells. This toxicity increased with the length of the carbon chain. A cell viability close to 0 % was observed immediately at 20 ug/ml for dendrons bearing the longer hydrocarbon chains (C18 and C19) [199]. In another study, Ionic and non-ionic bis-HMPA (cell viability > 83 %) were shown to be significantly less toxic than PAMAM dendrimers (cell viability 10 – 68 %) for both mesenchymal stem cells and U251MG malignant glioma cells at a concentration of 0.5 mg/ml [205]. Interestingly, functionalization with negatively charged chains decreased toxicity for bis-HMPA dendrimers, whereas PAMAM functionalized dendrimers were shown to be even more toxic than the non-functionalized analogues [205].

Here, the cytotoxicity of the synthesized dendritic compounds, as well as their corresponding *siDNA* dendriplexes was assessed in a human osteosarcoma (U2OS) cell line. Cytotoxicity was evaluated in terms of alterations in cell metabolic activity via a resazurin-based assay.

For PEG-dendrimers, the concentrations evaluated ranged between 0.25 and 1.5 mg/mL (Figure 4.6a) — relatively high concentrations when comparing with previously mentioned literature on biodegradable dendritic molecules for gene delivery. For every PEG-dendrimer and concentration tested, cell metabolic activity was higher than 85 % after 24 h of contact. This was expected, considering the partial shielding of the cationic amino groups by PEG chains.

The toxicity of the corresponding bio- and non-biodegradable dendriplexes with *siDNA* was also explored. Only the higher N/P ratios were evaluated regarding cytotoxicity (80 and 160), which were thought to potentially be more toxic to the cells since they contain a higher amount of PEG-dendrimer and hence a higher density of positive charges. These N/P ratios are approximately equivalent to a PEG-dendrimer concentration of 0.5 and 1 mg/ml, respectively. Again, the metabolic activity of the cells incubated 24h with the dendriplexes was shown to be above 90 % for every condition (Figure 4.6b). These results reveal the suitability of this new family of biodegradable dendritic structures for biomedical applications.

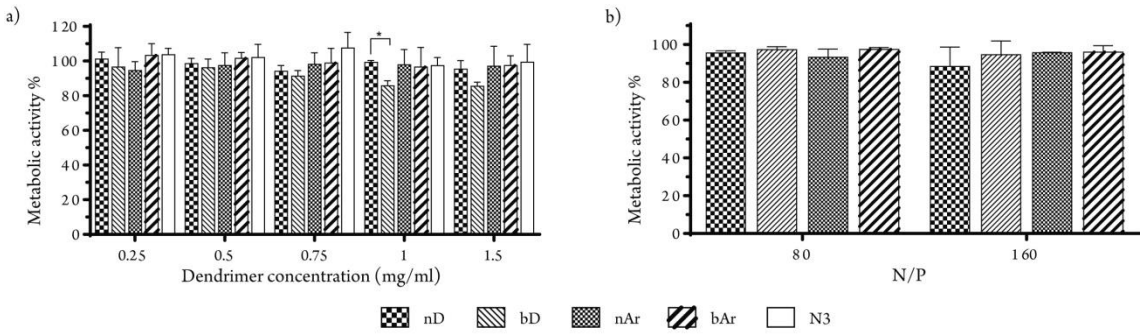


Figure 4.6 - Cytotoxicity evaluated by percentage of metabolic activity (resazurin assay) determined upon 24 h incubation of U2OS cells with: (a) biodegradable and non-biodegradable PEG-GATG dendritic structures: azide- and amine-terminated. (b) Dendriplexes at N/P 80 and 160. One-way ANOVA tests were used for statistical analysis. Significant differences: *p < 0.05.

4.3.3- Lethal concentration 50 (LC50)

The LC50 is a parameter that measures the drug concentration that kills half (50%) of the cell population in vitro and is expressed as a concentration, such as ug/ml or mg/ml.

Keeping in mind that the reported biodegradable PEG-bGATG are a newly developed synthetic material, it is important to assess the corresponding LC50. For that alterations in cell metabolic activity were measured upon incubation with dendrimers, using the resazurin assay.

To make sure cells did not reach confluence at the end point of the experiment, cells were seeded in order to have a confluence close to 50 % at the time of transfection. The concentrations tested ranged from 1.5 mg/ml, which is above the effective dose (1 mg/ml is equivalent to N/P 160), to 10 mg/ml.[1]

LC50 values were calculated based upon a linear regression analysis. Using the respective equations, the theoretical concentrations that would cause a 50 % decrease in cell metabolic activity were determined.

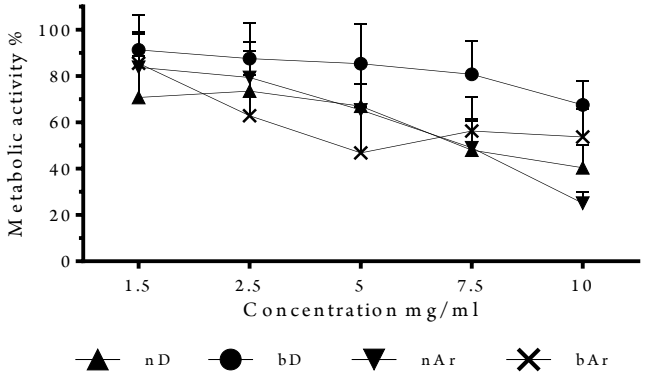


Figure 4.7 - Lethal Concentration 50 (LC50) for PEG-GATG dendritic block copolymers.

[1] For the lower concentration tested (1.5 mg/ml), nD presents high levels of toxicity (70%) when comparing with the first cytotoxicity experiments (Figure 4.6). This difference is most likely related with the mentioned lower cell density used for these experiments, which make the cells more vulnerable to the effects of exogenous compounds.

For biodegradable PEG-bGATG dendritic block copolymers, the LC50 was not possible to calculate, since those molecules did not cause a decrease of metabolic activity higher than 45 % (Figure 4.7). Moreover, the values obtained for PEG-bGATG-Ar did not follow a linear regression. From Figure 4.7 it can be concluded that PEG-bGATG-D was shown to be the less toxic PEG-dendrimer, with a cell viability of almost 70 % for the highest concentration tested, while PEG-bGATG-Ar showed values around 55 %. The non-biodegradable structures had values around 40 % (nD) and 25 % (nAr) (Figure 4.7).

In the case of non-biodegradable dendritic compounds, PEG-GATG-D and PEG-GATG-Ar, showed LC50 values of 7.9 mg/ml and 5.6 mg/ml, respectively (Table 4.1). Contrary to the first cytotoxicity experiments (Figure 4.6), where no difference was observed, here aromatic-terminated PEG-dendrimers are shown to be more toxic than the diamine analogues.

At the first look these results are unexpected, since a higher toxicity would be predictable for the structure with a higher density of positive charges, the diamine-terminated PEG-GATG. However, due to the hydrophobic nature of the aromatic group, there can be undetermined interactions with lipidic components of the cell (eg. cell membrane, intracellular vesicles) that can result in added toxicity effects.

Table 4.1 - Lethal Concentration 50 (LC50) for PEG-GATG dendritic block copolymers.

PEG-Dendrimer	Lethal Concentration 50 (LC50)
PEG-GATG-D	7.9 ± 2.3 mg/ml
PEG-bGATG-D	--
PEG-GATG-Ar	5.6 ± 1.5 mg/ml
PEG-bGATG-Ar	--

4.3.4 – Hemolytic activity

Several studies have reported that cationic dendrimers interact with RBCs, causing lysis, and subsequently Hb release [114, 163]. Again, this parameter needs to be addressed before exploring their efficacy as NA vectors, since in most cases nanoparticles are directly administered in the bloodstream. Additionally, some reports have suggested that aggregation of dendriplexes and erythrocytes could cause embolism or thrombosis [211]. Last but not least, the interaction between RBCs and erythrocytes diminishes the circulation half-life of the later, which hampers their efficiency [193].

In the present work, the hemolytic toxicity of the biodegradable amine-terminated PEG-bGATG was investigated by mixing both PEG-dendrimers and dendriplexes with a RBC suspension followed by 1h incubation at 37 ◦C. Fluorescence was further measured using a micro-plate reader. The conditions tested were the same as for the cellular toxicity. Thus, concentrations ranging from 0.25 to 1.5 mg/ml and N/Ps of 80 and 160 were tested for dendrimers alone and dendriplexes, respectively. N/Ps of 80 and 160 were chosen as they display a higher density of positive charges.

According to the results, both PEG-bGATG dendrimers are above the limit for hemolysis percentage, which is set at 10 % (Figure 4.8) [193, 212]. In all cases, the percentages of Hb release were above than 10 %, except for PEG-bGATG-Ar/*siDNA* dendriplexes at N/P 80, which was set around 9.3 %. Moreover, at every concentration, except at 1 mg/ml, diamine-

terminated PEG-dendrimers seem to be more toxic than the aromatic-terminated, which is expected since they have a higher number of positive charges that will promote a higher interaction with RBCs. Interestingly, this difference is not observed for dendriplexes. Complexation of PEG-dendrimers with nucleic acids leads to a reduction in cytotoxicity due to a partial shielding of cationic charges [123, 163]. These results are in agreement with previous reports stating that dendriplexes are less toxic than dendrimers [187, 213, 214].

The unexpected higher Hb release values obtained in this experience could be related with the fact that both dendrimers and dendriplexes are prepared in water, which upon mixing with erythrocyte solution (PBS) will increase the osmotic pressure of the cells, promoting lysis. Moreover, it is important to mention that the high values could also be related with the fact that the used protocol was optimized to test non-cationic nanoparticles with concentrations up to 0.5 mg/ml. These two factors (concentration and charge) can severely influence particle/RBC interaction. Therefore, the protocol will be optimized for cationic particles and further studies on RBC lysis will be carried out.

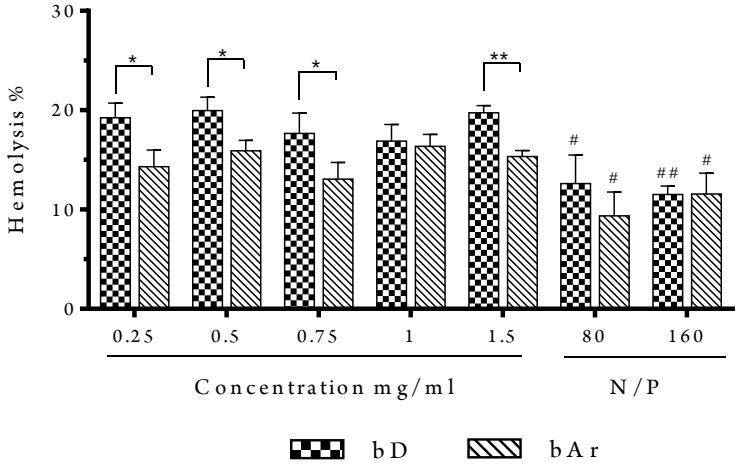


Figure 4.8 - Percentage of RBCs lysis after 1h incubation at 37°C with biodegradable PEG-bGATG dendritic block copolymers and their corresponding dendriplexes. Concentrations tested for PEG-dendrimers ranged from 0.25 to 1.5 mg/ml. For dendriplexes N/P ratios of 80 and 160 were tested. Independent t-tests were used for statistical analysis within concentrations. Significant differences: *p < 0.05 and **p < 0.01. # significant differences between N/P and corresponding concentrations (N/P 80 ≈ 0.5 mg/ml and N/P 160 ≈ 1 mg/ml)

4.3.5- Internalization efficiency

The ability of the developed dendriplexes to complex and/or to cross the cell membrane was carefully investigated by flow cytometry, confocal microscopy and imaging flow cytometry (ImageStream). U2OS/GFPLuc cells were incubated during 24 h at 37º C with the corresponding dendriplexes prepared with *siDNA* labeled with a cyanine fluorescence marker (Cy5).

For every dendriplex, flow cytometry shows an increase in Cy-5 fluorescence intensity when comparing with non-treated cells (red trace, Figure 4.9a). A higher intensity is observed as the N/P ratio increase, which is probably related to an increasing stability of the nanoparticles.

For all developed PEG-dendrimers, the percentage of positive cells for each condition tested was always above 95 %.

Regarding biodegradability, the difference between Lipofectamine and biodegradable dendriplexes is smaller than with the non-biodegradable analogues. Comparing PEG-dendrimers by their terminal functional groups (diamine vs benzylamine), the later have the highest Cy5 fluorescence intensity, especially PEG-bGATG-Ar with fluorescence values (at N/P 80 and 160) closer to the golden standard *in vitro* transfection reagent, Lipofectamine® 2000 (L2k). This is probably explained by the extra hydrophobic interactions between the dendriplex and the cellular membrane, which can promote a higher uptake as previously reported by Juliano *et al.* [214]. Kono and co-workers also showed that the surface modification of PAMAM dendrimers with hydrophobic moieties improved gene transfer efficiency due to stronger interactions between the plasma membrane and the dendrimer [215]. Thus, ordering by fluorescence intensity: L2k > bAr > bD > nAr > nD.

Confocal microscopy images, obtained by visualization of U2OS cells expressing EGFP-Luciferase upon incubation with biodegradable dendriplexes during 24 h, confirm cellular uptake (Figure 4.9b). The intracellular dotted-like Cy5 fluorescence pattern might be indicative of endosomal uptake of the dendriplexes (white arrows, Figure 4.9b). The increased stability of the PEG-bGATG-Ar dendriplexes (bAr) and the presence of favorable hydrophobic interactions with the cell's lipid membrane, imparted by the extra aromatic moieties, justifies the enhanced internalization of *siDNA*.

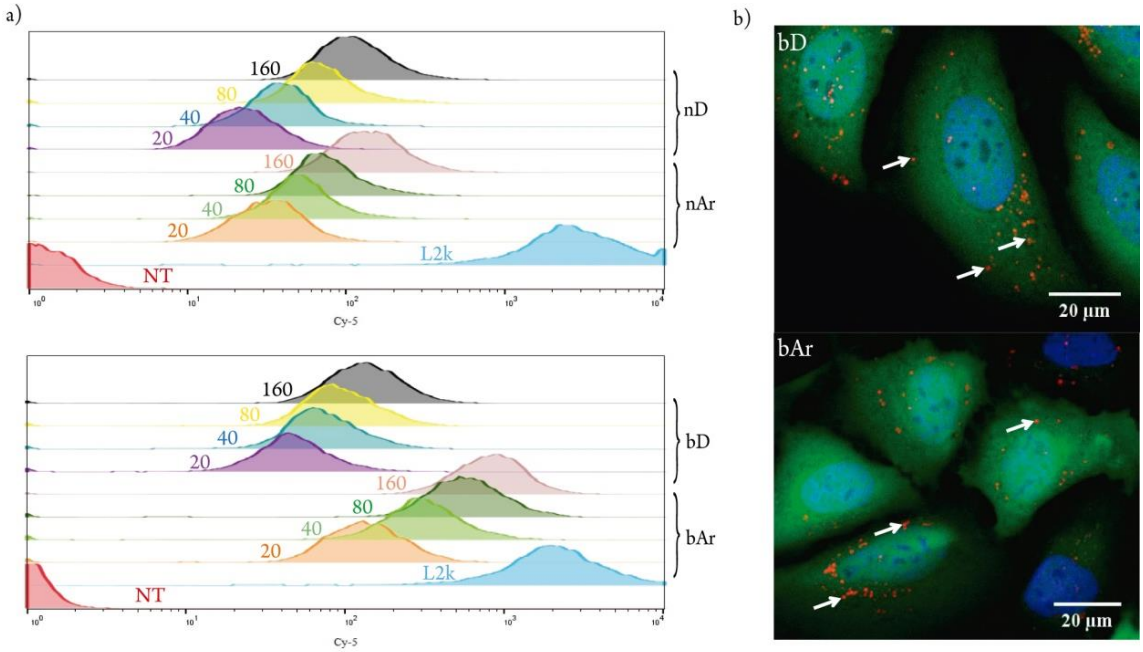


Figure 4.9 - Cellular association of dendriplexes. (a) Flow cytometry characterization at different N/Ps. (b) Confocal microscope images (z-stacks) for bD and bAr at N/P 160. Dendriplexes containing Cy5 labeled siDNA (Cy5-siDNA) were incubated for 24 h with U2OS cells at a final siRNA concentration of 0.1 µM. Blue: Nucleus (Hoechst 33342); Green: Cytoplasm (GFP); Red: Dendriplexes (Cy-5). Scale bar 20 µm.

Whereas traditional flow cytometry does not allow the determination of the localization of nanoparticles associated to the cells, confocal microscopy, while assuring spatial resolution, is time consuming, expensive and lacks the statistical strength of flow cytometry [216, 217]. Thus, to further quantify and characterize the internalization of the dendriplexes,

imaging flow cytometry was performed. This new technique is a high-throughput technology that brings together the quantification capacity from traditional flow cytometry with image acquisition [216, 217].

First, the total fluorescence intensity values in the cytoplasm were computed for every studied PEG-dendrimer (Table 4.2). The obtained results are in agreement with the results obtained for flow cytometry analysis shown in Figure 4.9a. For both groups (bio- and non-biodegradable), the aromatic-terminated PEG-dendrimers seem to be more efficient in cellular internalization than the diamine-terminated ones, showing again the relevance of the hydrophobic interactions. Additionally, these results confirm that biodegradable PEG-dendrimers are in general best performing, with more than a 2- and 3-fold increase in mean and median intensity when comparing with non-biodegradable PEG-GATG (Table 4.2). As explained before, the higher internalization rates could be related with a more effective nanoparticle packaging, due to the more hydrophobic arms of the bGATG monomers, comparing with the highly hydrophilic arms of the GATG monomers.

Moreover, for every dendriplex tested, the number of Cy-5 positive cells was always above 97 %, which also confirms the values obtained for flow cytometry.

Table 4.2 - Fluorescence values for Cy-5 labeled *siDNA* obtained by Imaging Flow Cytometry.

PEG-dendrimer	**Cy-5 Fluorescence Intensity in the cytoplasm**		
	Mean	**Median**	**Maximum**
PEG-GATG-D	9.0×10^3	6.6×10^3	4.1×10^5
PEG-GATG-Ar	1.3×10^4	9.3×10^3	7.3×10^5
PEG-bGATG-D	3.0×10^4	2.7×10^4	1.5×10^6
PEG-bGATG-Ar	3.6×10^4	3.3×10^4	4.1×10^5

After assessing the intensity values, the percentage of nanoparticle-loaded vesicles (NLVs) per cell was also evaluated. Cell images were taken in several different planes on the z axis and an image projection was created as mentioned in the materials and methods section. Three populations defining cells with low (< 1.5), medium (1.5-5.5) or high (> 5.5) number of vesicles per cell were determined for each condition tested (Figure 4.10). As mentioned in section 3.1.14 (Materials and Methods), these three groups were created based on the worst performing dendrimer, PEG-GATG-D. The number of NLVs per cell was plotted in a histogram accordingly to these values (Figure 4.11). Figure 4.10 is an example with the three defined categories (low, medium, and high) for the number of vesicle-loaded particles.

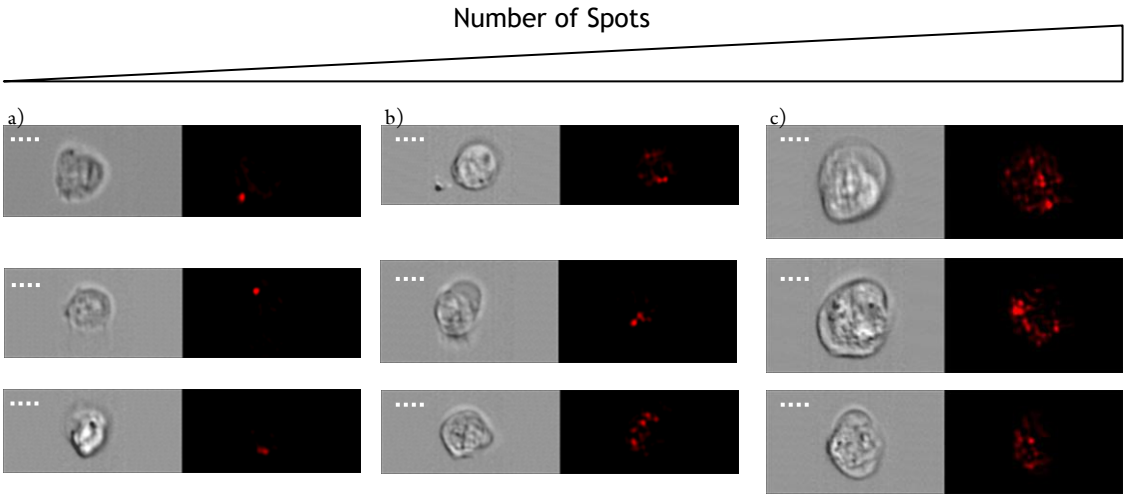


Figure 4.10 - Nanoparticle-loaded vesicles (NLV). Extended Depth of Field images acquired by Imaging Flow Cytometry for PEG-bGATG-Ar/*siDNA* dendriplexes. Representative images for every category are shown in: a) Low spot count; b) Medium Spot Count; and c) High spot count. Gray background: bright field images; Black background: channel 5 images (Cy-5). Scale bar: 10 μm.

Twenty-four hours post transfection large differences were observed regarding the percentages for every group as shown in Table 4.3. Following the trend observed for cytoplasm Cy5-intensity values, PEG-bGATG-Ar was the best performing dendrimer as it displayed a higher percentage of cells with high number of NLV (44 %). This result shows that PEG-bGATG-Ar is capable of introducing Cy-5 labeled *siDNA* into cells more efficiently than the remaining dendrimers (Table 4.3).

Moreover, the higher efficiency of biodegradable PEG-dendrimers comparing with their non-biodegradable analogues arises from these results.

Table 4.3 - Nanoparticle-loaded vesicles (NLVs).

PEG-dendrimer	**Nanoparticle-loaded Vesicles**		
	Low (<1.5 spots)	**Medium (1.5 - 5.5 spots)**	**High (>5.5 spots)**
PEG-GATG-D	23 %	62 %	15 %
PEG-GATG-Ar	13 %	63 %	24 %
PEG-bGATG-D	16 %	51 %	33 %
PEG-bGATG-Ar	4 %	52 %	44 %

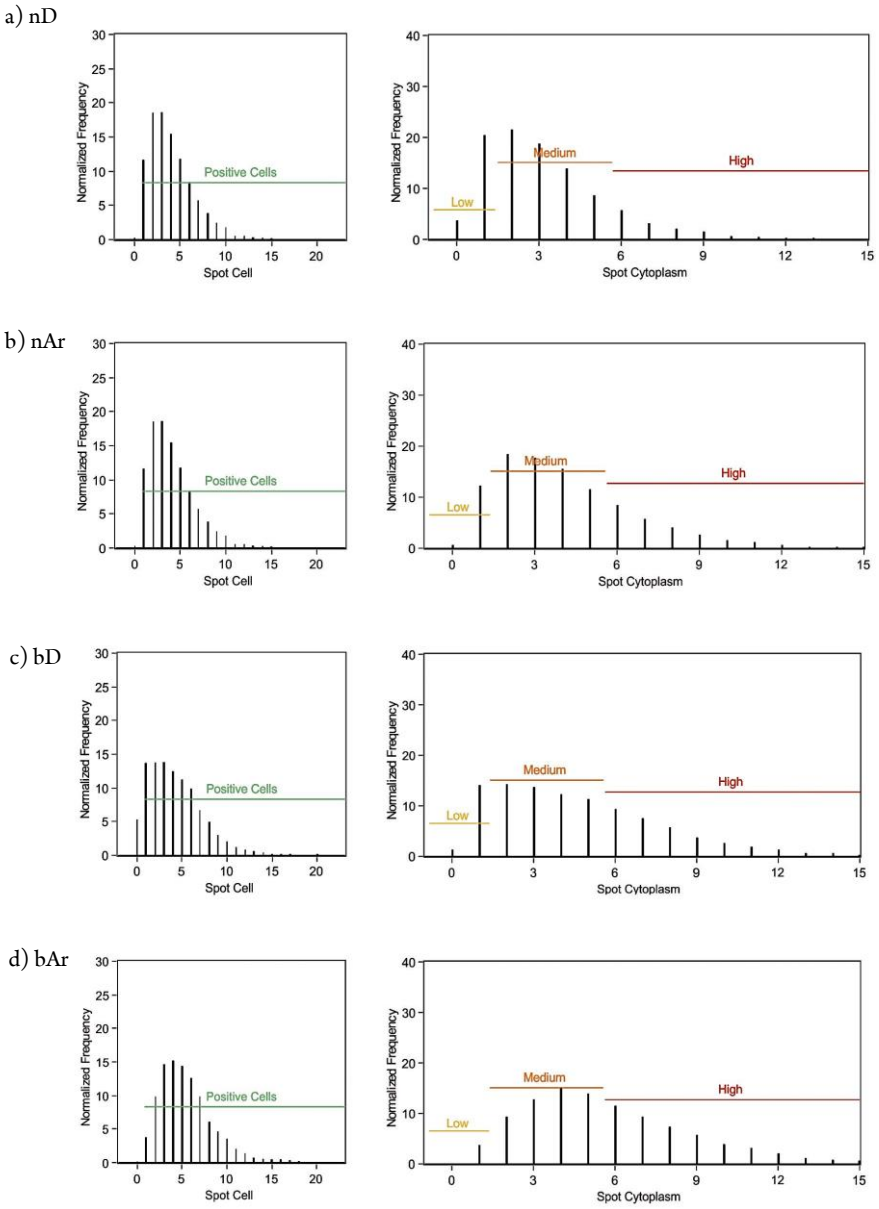


Figure 4.11 - Nanoparticle-loaded vesicles (NLV) per cell. a) PEG-GATG-D, b) PEG-GATG-Ar, c) PEG-bGATG-D, d) PEG-bGATG-Ar. Left column: Spot count for the cell mask. Right column: Spot count for the cytoplasm mask. Green: region for Cy-5 positive cells (membrane and cytoplasm); Low: region for low spot count cell (cytoplasm); Orange: region for medium spot count cells (cytoplasm); Red: region for high spot count cells (cytoplasm).

4.3.6 – Transfection efficiency

Finally, the ability of the developed PEG-dendrimers to mediate gene silencing was tested. For that, U2OS cells stably expressing the fusion protein EGFP-Luciferase were incubated for 24 h at 37 ºC with dendriplexes containing anti-GFP siRNA. Transfection efficiency was measured as a decrease in luciferase activity, which was then expressed as a percentage of the enzyme's activity measured for non-treated cells.

First, to explore the N/P effect, the transfection efficiency for PEG-bGATG-D dendriplexes at several N/P was studied (Figure 4.12a). The first results showed no decrease in the luciferase activity (checkered pattern). Thus, chloroquine, a disruptor of endosomal

vesicles that acts through a buffering mechanism promoting an increase on vesicle osmotic pressure [218, 219], was added 24 post-transfection in order to explore if endosomal escape was proving to be one of the limitations steps. With chloroquine a decrease in luciferase activity was observed for N/Ps ≥ 40, which was then shown to increase in an N/P dependent manner (around 70 and 80 % silencing at N/P 80 and 160, respectively).

Then, we decided to study the transfection efficiency for all the developed dendritic carriers at N/P 160 that showed to be the best performing ratio for PEG-bGATG-D. Additionally, the study was carried under both conditions: presence and absence of chloroquine (Figure 4.12b)

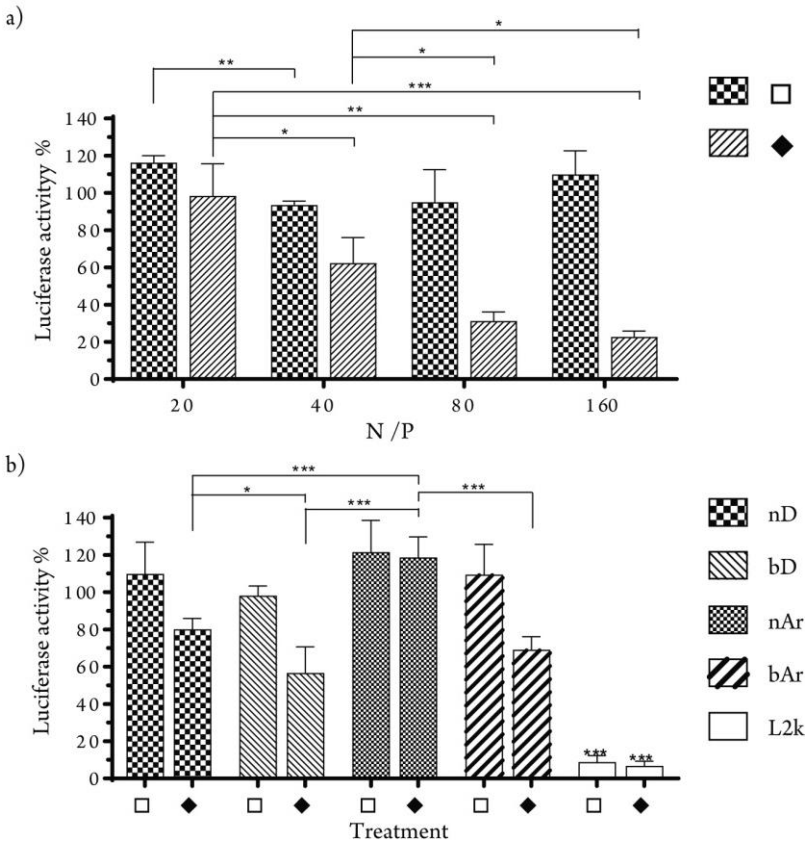


Figure 4.12 - Percentage of Luciferase activity upon 72 h post-transfection for: a) PEG-bGATG-D (bD) dendriplexes at different N/P. b) Bio- and non-biodegradable PEG-GATG dendriplexes: nD, bD, nAr, bAr at N/P 160. Experiments in the absence (□) and presence (◆) of chloroquine (CQ). One-way ANOVA tests were used for statistical analysis. Significant differences: *p < 0.05, **p < 0.01 and ***p < 0.001.

In presence of chloroquine, silencing was observed for all studied dendritic carriers, except for non-biodegradable PEG-GATG-Ar. Biodegradable PEG-bGATG dendrimers showed promising results as expected with silencing activities around 40 % and 30 %. We suggest that due to presence of the degradation points at the branches that bind directly to the siRNA, they promote a higher siRNA release, thus contributing to the improvement of the transfection efficiency when comparing with the non-biodegradable PEG-GATG structures. Despite PEG-bGATG-Ar ability to delivery higher siRNA amounts into cells (Figures 4.9 and 4.11 and Tables 4.2 and 4.3), a similar silencing effect to PEG-bGATG-D was observed. This could be related to the previously shown higher stability of PEG-bGATG-Ar dendriplexes, which was demonstrated by its higher ability to protect *siDNA* from enzymatic degradation

(Figure 4.5). Therefore, it is reasonable to hypothesize that they have a lower ability to intracelullarly release siRNA under these conditions.

For experiments without chloroquine there is an increase in luciferase activity (Figure 4.12). Thus, a control experiment was carried out by testing the effect of the PEG-dendrimers in luciferase activity. Again, it is observed that PEG-dendrimers/anti-GFP siRNA dendriplexes lead to a significant decrease in luciferase activity (≈ 50 %) when incubated with chloroquine (Figure 4.13).[2]

Regarding the PEG-dendrimers, a luciferase activity close to 125 % for PEG-bGATG-D and 130 and 200 % for PEG-bGATG-Ar without and with chloroquine is observed, respectively (Figure 4.13). Thus, one could hypothesize that the dendrimers may influence gene expression on their own.

In fact, the influence of different lipidic and polymeric delivery agents alone in endogenous gene expression has already been reported [220-223]. Oligofectamine and lipofectin were shown to alter the expression of different genes related with several cellular pathways [224]. Moreover, gene expression profiles are known to be dependent on the nature and structure of the delivery vehicle and whether it is complexed with nucleic acids [222, 223]

Likewise, studies have reported that dendrimers are able to intrinsically modify endogenous gene expression. Omidi *et al.* used microarrays to show that PPI dendrimers alone could influence important cellular pathways such as apoptosis, cytokine signaling and proliferation [171]. A few other reports have been made on the influence of dendrimers on endogenous cellular pathways by gene up- and down-regulation [172, 174-177].

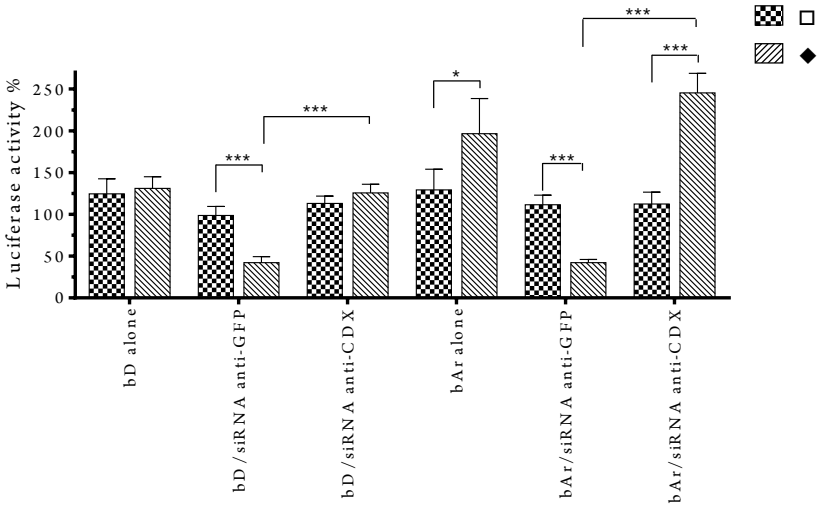


Figure 4.13 - Controls for luciferase activity upon 72 h post-transfection for biodegradable PEG-bGATG dendrimers. Experiments in the absence (□) and presence (◆) of chloroquine (CQ). One-way ANOVA tests were used for statistical analysis. Significant differences: *p < 0.05, **p < 0.01 and ***p < 0.001.

[2] The differences observed in silencing activity for bD at N/P 160, between experiments (Figures 4.12 and 4.13) can be caused by the instability of the GFPLuc integration on the U2OS/EGFPLUC stable cell line which can vary with the cell passage number. This leads to some variation on the EGFP-LUC expression levels which in turn will affect the total silencing effect achieved by certain siRNA concentration.

Delivery materials with encapsulated siRNA were shown to promote diverse "off-target" effects by simply introducing them into different cellular compartments [222]. To confirm that the luciferase activity decrease was specific for the anti-GFP siRNA, transfection experiments with dendriplexes prepared using a siRNA molecule non-complementary to GFP (anti-CDX siRNA).

From these experiments, it can be concluded that the decrease in luciferase activity obtained with anti-GFP siRNA was not owed to an "off-target" effect. In fact, an increase up to 2.5-fold in luciferase activity was shown with PEG-bGATG-Ar/anti-CDX siRNA dendriplexes, even in the presence of chloroquine. We hypothesize that this increase is most likely related with the bAr dendritic compound itself as previously mentioned, as for PEG-bGATG-Ar we see an increase of 2 fold (Figure 6.3.6.2).

To conclude, the developed biodegradable PEG-dendrimers were shown have suitable physicochemical characteristics for siRNA delivery. Additionally, they promoted higher transfection efficiency than their non-biodegradable analogues, due to the ester bonds within their structure. Upon hydrolysis of these degradation points, the siRNA molecules are more easily released, hence increasing their bioavailability, and promoting a higher silencing activity.

Chapter 5 - Conclusions

Gene therapy requires the detailed design of delivery vehicles that can safely and efficiently deliver nucleic acids into cells with minimal toxicity. Dendrimers are being proposed as promising candidates for non-viral nucleic acid delivery due to their unique characteristics, such as: defined and globular architecture, low polydispersion and the presence of several terminal groups that allow multifunctionalization.

However, most of the dendritic compounds developed and reported so far have been associated with high levels of toxicity, hence hampering their translation to clinical applications. Toxicity is often associated with their cationic profiles, but also with long term tissue accumulation. While PEGylation and other surface decorations are able to mask the positive charge of the compounds, it doesn't prevent tissue accumulation of synthetic materials. Thus, several teams are focusing on the synthetic challenge of developing biodegradable dendritic structures that should degrade under physiological conditions and be more easily excreted from cells and tissues. However, owing to undesired and premature degradation, there are still a very reduced number of studies where biodegradable dendrimers are reported for biomedical applications.

In this work, we evaluated the performance of a new family of biodegradable and biocompatible PEG-bGATG dendritic block copolymers as siRNA delivery vectors.

The size, charge and morphology of the nanoparticles play an essential role on cellular uptake and transfection efficiency. Thus, these physicochemical characteristics are important aspects that need to be taken into account when developing delivery vehicles for gene therapy. Here, we have shown that the developed PEG-dendriplexes suitable features for this application: sizes between 140 and 200 nm, surface charge above 10 mV, globular structure and achieved complexation efficiencies above 90 % for most of the tested conditions.

Moreover, the nature of the terminal groups was shown to affect dendriplex formation and complexation efficiency. Benzylamine-terminated PEG-dendrimers promoted the formation of more homogeneous dendriplexes and were also shown to have an increased cellular uptake, due to the additional hydrophobic interactions from the aromatic group.

Regarding biodegradability, the presence of ester bonds was shown to play a role in the cytotoxicity profiles for the tested PEG-dendrimers, as shown by the LC50 measurements. Additional, these degradation points were crucial for a better siRNA release from the dendriplexes, contributing to the significant improvement of the transfection efficiency when comparing with the non-biodegradable PEG-dendriplexes.

In conclusion, the set of results presented herein are encouraging since we were able to modulate gene expression with the first biodegradable dendritic compounds described for siRNA delivery.

Chapter 6 - Future Perspectives

The ideal NA delivery vehicle should be able to overcome every limiting step in the extra and intracellular environment in order to achieve an efficient gene delivery. The developed PEG-dendrimers have shown a limited endosomal escape. Thus, one of the main aims for future work is to improve this aspect by surface modification of the dendrimer. The introduction of buffering moieties such as imidazole, is expected to increase the buffering capacity of the nanoparticles and enhance endosomal destabilization.

Further studies on internalization kinetics, intracellular trafficking, as well as "off-target" effect studies could be made in order to better characterize the delivery profiles of the reported PEG-dendrimers.

Finally, *in vivo* studies on dendrimer cytotoxicity and biodistribution should be performed to test the advanced hypothesis that biodegradable dendrimers over perform non-degradable analogs in terms of biocompatibility and avoid long-term cell/tissue accumulation.

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